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# THE AMERICAN JOURNAL OF ANATOMY

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## EDITORIAL ANNOUNCEMENT

With the present volume **THE AMERICAN JOURNAL OF ANATOMY** enters a new phase of its history. A transfer of actual ownership occurred some months ago, but only now have the results of this change actually expressed themselves. The first most noticeable effect is a partial transformation in the personnel of the Board of Editors of **THE JOURNAL**, and a word of explanation and statement of intentions seem proper.<sup>1</sup>

**THE JOURNAL** was originally founded by a small group of Anatomists who up to the past year retained ownership and the responsibility for its editorial management. These trustees, along with other members of their Editorial Board, a number of years ago delegated the business arrangements and the publication of **THE JOURNAL** to a central office established by The Wistar Institute of Anatomy. The advantages of this arrangement, both in facilities for publication and distribution of **THE JOURNAL**, have been manifold. The trustees realizing these advantages finally deemed it proper to actually transfer their ownership of **THE JOURNAL** to the Wistar Institute of Anatomy, with the understanding that the editorial management and scientific policy would be arranged for between The Wistar Institute and the American Association of Anatomists.

An agreement has been reached between The Wistar Institute and the Association of Anatomists whereby a Journal Committee of the Anatomists has been established, and a proper cooperation of the Institute and the Association in the future affairs of **THE AMERICAN JOURNAL OF ANATOMY** is assured.

*In the scientific policy and purposes of **THE JOURNAL** no change has occurred.* The aims and ambitions of the present editors are none other than those of their predecessors. We purpose

<sup>1</sup> The articles appearing in this number, and a few others yet to be published, were received by the former Editors.

only a fresh effort to maintain the degree of excellence attained by the Founders of THE JOURNAL and its first Editorial Board. In accomplishing this aim progressive changes must frequently be made.

As a present means of facilitating promptness in publication and allowing space for a larger number of articles, authors are urged to condense their communications as far as possible. Many papers may readily be improved by shortening the text, and by this curtailment they may often become more widely read. We might initiate a new era of direct and concise scientific style and lend much to the value of THE JOURNAL. As the science of Anatomy advances from the purely descriptive to the more analytical and experimental phase, it may further adopt a briefer method of recording its discoveries. The condensed style may not always be possible, but where it can be used the apprehension of the main points at issue is greatly facilitated.

The precarious situation in which scientific publication abroad now finds itself, becoming almost impossible in certain cases, should not only impress us with our more fortunate position in this regard, but should stimulate an improvement both as to quality and directness of style in scientific writing.

No doubt many other possible improvements in the character and usefulness of THE JOURNAL will present themselves as time goes on, but a realization of these must in all cases depend upon the sincere efforts and cooperation of the Anatomists whom THE JOURNAL was founded to serve.

The Editors pledge their most vigorous efforts to the duty they have undertaken, and earnestly desire the support of those interested in the future development of Anatomical Science in any or all of its phases.

CHARLES R. STOCKARD,  
*Managing Editor.*

THE AMERICAN JOURNAL OF ANATOMY, VOL. 29, NO. 1  
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Resumen por los autores, William H. F. Addison  
y Harold W. How  
University of Pennsylvania y Wistar  
Institute of Anatomy

El desarrollo de los párpados de la rata albina, hasta la terminación de la disyunción.

Los autores han seguido paso a paso el desarrollo de los párpados, desde el momento en que aparecen por primera vez hasta el tiempo de la disyunción. Los párpados se fusionan durante el décimo-octavo día de la vida fetal (cuatro días antes del nacimiento) y se separan durante al décimo-cuarto al décimo-séptimo día después del nacimiento. Midiendo el epitelio de unión se encuentran tres estados desde la fusión hasta la disyunción de los párpados: (1) Fusión activa, durante la cual la dimensión conjuntivo-epidérmica de la unión epitelial aumenta de extensión; (2) la unión estacionaria; (3) la separación gradual. El primer estado comprende desde la primera unión de los párpados por una membrana epitelial hasta cuatro días después del nacimiento, el segundo hasta los seis o siete días y el tercero hasta la terminación de la disyunción.

El principal factor en la separación del epitelio de unión durante el tercer estado es el proceso de queratinización, que continúa hacia dentro desde las células epidérmicas superficiales del rafe hasta que alcanza el lado conjuntival de este último. Otros factores concurrentes que juegan un papel menos importante en el proceso de la disyunción son los centros de queratinización en; (1) los folículos pilosos, que se han originado en el epitelio de unión: (2) Los conductos de las glándulas tarsales, y (3) el surco conjuntival, un poco antes de la disyunción. El periodo de unión de los párpados está relacionado temporalmente con: (1) La maduración de la piel, desde la primera aparición de los folículos de los pelos ordinarios hasta el recubrimiento completo del cuerpo por los pelos, y la erupción de los pelos en los bordes de los párpados, y (2) Con la maduración de la retina, desde una condición embrionaria temprana hasta que presenta todas las capas definitivas de la retina funcional.

Translation by José F. Nonides  
Cornell Medical College, New York

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## THE DEVELOPMENT OF THE EYELIDS OF THE ALBINO RAT, UNTIL THE COMPLETION OF DISJUNCTION

WILLIAM H. F. ADDISON AND HAROLD W. HOW

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Anatomy*

THREE TEXT FIGURES AND TWO PLATES (THIRTEEN FIGURES)

When the eyes are first formed no lids have yet appeared. Then, following the formation of the lids, there is a definite period when they are fused together by their epithelial margins. This period varies with the different mammalian species, but is more or less constant for the individuals of any one species. In all, the time of formation and fusion occurs during fetal life, while the subsequent separation or disjunction of the lids occurs, in some species, during a later stage of fetal development, and in others it does not occur until after birth. The time of disjunction is related, in general, to the state of development of the entire organism, and in particular to the development of the retina.

In the form we have studied, the albino rat, the gestation period averages twenty-two days, and it is during the eighteenth fetal day that the eyelids fuse. After birth the lids remain closed usually for fourteen to fifteen days, and sometimes for one to two days longer. In our study, we have followed the histological changes involved in the fusion and disjunction of the lids, and have attempted to correlate the time period of this process with the development of related organs.

### MATERIAL AND METHODS

For the histological study sections were prepared of the eye regions of fetuses from the seventeenth to the twenty-first days of gestation, and of young animals from birth to fifteen days, at



one-day intervals: For stages up to one week old paraffin embedding was satisfactory, but after the appearance of the hairs double embedding in celloidin and paraffin gave better results. In preparing for embedding the material to show the later stages, especially from seven days onward, sometimes the lens was removed, and in some stages only the eyelids were embedded. For routine staining hematoxylin and eosin were used.

#### *Seventeen-day fetus*

At this stage there are protruding ridges in the position where the lids are about to develop, but one cannot say, as yet, that the lids have been formed. As a consequence, the eye is uncovered (fig. 1) and shows plainly on inspection. In sections across the eye region the ridges are seen, as shown in figure 2. When examined under the microscope, both the epithelial cells of the skin of the head and the epithelial cells covering the ridges above and below the eye are found to be two or three rows in thickness, but the epithelium at the margins of the ridges is thicker than the epithelium elsewhere. This is because the cells of the basal row have here a greater height than elsewhere, and are of a distinctly tall columnar shape, while the cells of the basal row in regions near by are of a lower columnar type. The cells of the superficial row or rows everywhere are seen to be flattened in form. Of course, at this stage growth is active everywhere and mitoses are abundant, but at the time under consideration the epithelium at the margin of the ridges has the appearance of being the site of relatively greater activity than in the skin near by. The margin of the ridge constitutes, in fact, an apical line of growth.

#### *Eighteen-day fetus*

Inspection of fetuses at this stage (fig. 3) shows that the eyelids have developed rapidly in the preceding twenty-four hours and now cover the eyeball completely. Examination of sections through the eye region (fig. 4) usually shows that the two lids have already fused together by their epithelial margins.

This was found to be the condition in sections of fetuses from three different litters, except in the case of two individuals. In one of these (fig. 5) the development of the lids was less advanced and fusion had not everywhere taken place. The illustration is taken from the middle region of the eyelids, and laterally to this section the epithelial margins are in much closer approximation. Evidently in the albino rat, as in other species, the middle region of the lids is the last to completely fuse. As the figures show, the two lids are of somewhat different proportions, the upper being thicker and shorter and the lower more compressed and longer.

Microscopic examination of sections, from which figure 5 was taken, shows that the skin epithelium now has four or five layers of cells, the basal row being columnar in type, the remaining ones flattened. On the margin of the eyelid the epithelium is much thicker than elsewhere, and forms, in section, a conical-shaped projection about  $100\mu$  in length. The basal row of cells is taller than on the neighboring skin and there are many more rows of superficial cells. These superficial cells are rounded or polyhedral for the most part, arranged with their longer axes at right angles to the margin of the lids, and are stained lightly. On the external surface of the epithelial projection, however, the cells are more flattened and are darkly staining, as in the superficial cells of the epithelium of the skin.

In another series of sections, from a fetus about eighteen days of age, where the lids were in practically the same stage of development as in this, the part played by the epithelial portion of the eyelid in the primary closure is seen still more distinctly. In the middle of the lid the epithelial margins are still separated by a short space, but immediately adjacent to this region the epithelium forms a thin narrow connecting membrane  $350\mu$  in extent, between the mesenchymal portions of the two lids. The plane of section cuts the lids slightly obliquely, but in this case, as in the preceding, the evidence is conclusive that the epithelium grows out actively beyond the mesenchymatous portions of the lid, to bridge the gap between the two approaching lids. After the primary union is established, the framework

structures of the lids continue to grow and to approximate until the epithelium forms only a narrow band between the two lids. As the two lids come together, it appears that the greater part of the intervening epithelium is pressed aside. This is to be seen as little irregular groups of epithelial cells attached to the junction epithelium, either on the outer side or on the conjunctival side (figs. 4 and 6). In figure 4 the epithelium between the two lids measures  $63\mu$ . Here the basal row of epithelium on each lid margin is again columnar, while the intervening cells are rounded and vesicular. The length of the band of epithelium measured in the direction of the line of fusion is about  $90\mu$ .

At this period, as before, the vibrissae buds of the face are distinct, but now for the first time other hair buds are beginning to develop in the skin, and occasionally one is found on the surface of the lid itself. The muscle fibers in the lid are plainly to be seen and the mesenchyme is distinctly more condensed on the conjunctival side of the lid.

#### *Nineteen-day fetus*

At this stage the proportions of the band of epithelial cells forming the junction have already become somewhat altered (fig. 6). By the growth of the opposing mesenchymatous structures the junction epithelium now measures only about  $50\mu$  from the basement membrane of one lid to that of the other. While in the direction from the conjunctival surface to the outer surface it has doubled in extent and measures about  $180\mu$ . This junction epithelium consists of a basal layer of columnar cells resting on the mesenchymatous portion of each lid, and three or four irregularly disposed rows of pale-staining, rounded, vesicular cells intervening. Both on the inner and outer sides of the junction epithelium are seen clumps of epithelial cells (fig. 6, *ep*) which have been pressed aside by the opposing growth of the two lids.

#### *Twenty-day fetus*

The cells of the epithelium on the outer surface of the lids are now arranged in three strata. The basal layer is columnar,

deep-staining, and shows abundant mitoses. The intermediate layer is composed of one or two rows of pale, vesicular cells, while on the surface are two or three rows of flattened cells showing the beginning formation of keratohyalin granules. The junction epithelium consists of the same three varieties of cells, but arranged in the following manner. On the basement membrane of each lid margin is a layer of columnar epithelium, and, filling the intervening space between these two layers, are rounded cells of a pale-staining, vesicular appearance. While on the outer surface of the junction epithelium are two or three layers of flattened, dark-staining cells, containing keratohyalin granules, and continuous with the similar cells of the adjacent skin surface. The progress of the keratinization process in the superficial cells of the epithelial junction will constitute one of the chief features to observe in watching the changes leading up to the final disjunction of the lids.

At the outer margin of the lids are found buds of hair follicles, but as yet no distinct proliferation of epithelium to form the tarsal glands has appeared. The muscle tissue of the lids is now more apparent and the mesenchyme is becoming more differentiated, so that it has a denser texture immediately beneath the epithelium both of the skin and of the conjunctiva with a looser texture intervening. Examination with the binocular microscope shows that a few of the vibrissae of the snout region have already advanced through the epidermis and are now apparent on the outside of the skin.

#### *Twenty-one day fetus*

At this age the beginning of the tarsal glands is shown by the proliferations of epithelial cells at the inner angle of the margin of the eyelid (fig. 7, *t*). Thus the tarsal glands follow closely, in point of time, the appearance at the outer angle of the eyelid of the hair-follicle buds which are noted at the twenty-day stage. This was also the order of appearance observed by Ewetsky ('79) in fetuses of domestic cattle. He saw the anlage of the cilia at the 6-cm. stage and of the tarsal glands at the 8.5-cm. stage.

The hair-follicle buds are increasing in number and are seen in various early stages of development. The oldest ones have a papilla at their basal ends, but none yet show the hair shaft within. Many of the vibrissae follicles, however, are completely differentiated and contain a well-defined hair shaft. Examination of the outside of the head region shows that many vibrissae are protruding above the surface of the skin and that some of them are nearly 1 mm. in length.

Measurements of the junction epithelium, both at this and the preceding stage, show that the adjoining parts of the lids have continued to grow and that there is an increase in the dimensions of the junction epithelium as compared with the nineteen-day stage. Measured from the basement membrane of one lid to that of the other, it was  $54\mu$ , while in the conjunctiva-skin direction it was  $240\mu$ .

#### *Newborn rat*

In the newborn rat (fig. 8) the epidermis shows a differentiation into stratum germinativum, stratum granulosum, and stratum corneum. The stratum germinativum is composed of a basal layer of cells with deeply staining nuclei and of several rows of paler staining cells. The stratum granulosum is very distinct, by reason of keratohyalin granules in the cytoplasm of the cells (fig. 8, *k*), while the stratum corneum is composed of flat scale-like cells. At the junction the epithelium consists of cells similar, for the most part, to those in the stratum germinativum, i.e., a basal layer of cells with dark staining nuclei on each basement membrane and polyhedral vesicular cells intervening. On the outer surface of the junction, however, the stratum granulosum and the stratum corneum of the surface epithelium continue uninterruptedly.

The dimensions of the junction epithelium are found to be less than those at the preceding stage. This is to be associated, probably, with the change of environment at birth. The length from the conjunctival surface to the outer limit of the stratum granulosum measures  $180\mu$ , while at the narrowest point between the two lids it measures  $40\mu$ .

Inspection of figure 8 shows that there is a slight indentation of the outer surface of the junction epithelium, a feature which becomes progressively more marked at later stages. In the skin are more hair follicles than before. These are of various ages, some just beginning, others already differentiated and containing a hair shaft (fig. 8, *h*). But none of the hair shafts can be traced beyond the epidermis. The vibrissae on adjoining parts of the head are already well-developed structures, projecting as much as 3 mm. beyond the surface of the skin. The hair follicles of the outer angle of the lid, however, are mostly in a young condition, and only in the largest ones can be seen a developing shaft. The longest follicles measure  $175\mu$ . The tarsal glands at the inner angle are still short, solid, flask-shaped epithelial buds, measuring about  $70\mu$  in length. The voluntary muscle fibers in the lids are growing in size and now have their nuclei peripherally placed.

#### *Two-day rat*

Considerable advance is seen in the structures within the lids as compared with the condition in the newborn. The hair follicles are more numerous and the older ones have increased both in diameter and in length. Many of the largest follicles contain a hair shaft, and an occasional one of these begins to protrude through the epidermal surface. Those in the lids nearest the junction are, however, not so far advanced as those farther away from the junction, and while some of the former show beginning differentiation of the hair shaft, they are not yet fully formed. Midway between the follicles at the external angle of the lid, and the tarsal glands at the internal angle are seen very small buds of hair follicles which are just beginning to develop from the junction epithelium.

The junction epithelium now measures  $250\mu$  in length from the conjunctival surface to the outer epidermal surface, as compared with  $180\mu$  at birth. The diameter of the junction epithelium at its narrowest point is about  $30\mu$ . The indentation on the outer surface of the junction epithelium is slightly more



pronounced than before and both the stratum granulosum and the stratum corneum are a little thicker at that point than in the adjacent skin epidermis.

### *Three-day rat*

Although sections show that hair follicles are now a prominent feature of the skin of the lid and that many of them contain definite hair shafts, very few of the latter have yet appeared above the surface of this skin. Coincident with the differentiation of the hair shaft within the basal part of the follicle there is a keratinization process going on within the outer part of the follicle where it is continuous with the surface epithelium. This process results in the formation of a canal or outlet to the follicles, through which the developing hair shaft will grow. At this period the process is only partly completed in many follicles.

The most evident change in the junction epithelium is the slight deepening of the outer groove. Its measurements are  $250\mu$  in the conjunctival-epidermal length and about  $40\mu$  at its narrowest part between the two lids. The hair-follicles at the outer angle of the margin of the lids are in several stages of development. The longest, measuring 200 to  $250\mu$ , show a distinct papilla at the base and a growing shaft within the root sheaths. Near the mouth of the follicles are small oval out-buddings of epithelial cells to form sebaceous glands. Where these longest follicles are connected with the outer part of the junction epithelium, the cells in the line of future progression of the hair shaft have become more cornified and their nuclei are no longer apparent. These cells show the situation of the mouth of the follicle, and the stratum granulosum of the epidermis is seen to be dipping in to form the boundary of the mouth of the follicle. It is thus easy to be seen that the formation of the mouths of these follicles has a distinct effect upon the outer part of the junction epithelium in the way of tunnels being formed through it in preparation for the hair shafts growing out.

The tarsal glands now measure  $175\mu$  in length, whereas at birth they measured only  $70\mu$ .

*Four-day rat*

Hairs are beginning to show on the outside of the lid epidermis as well as on the other parts of the head and body, but these are barely visible on casual inspection with the naked eye, and in consequence the pink color of the skin is very slightly affected by the white color of the sparse short hair. In sections, however, hairs are readily found projecting from the longest follicles (fig. 9). At the margin of the lids it is noticeable that the follicles are not as far advanced as in other parts of the lids. In figure 9, *h*, are shown hair follicles still in an early stage of development from the junction epithelium. In some of the follicles near the margins of the lids, as at the three-day period, there is a beginning differentiation to form the shaft at the basal end, and there is a keratinization process going on in the outer ends of the follicles leading to their canalization. Where this cornification is seen there the stratum granulosum of the epidermis dips inward and the stratum corneum of the epidermis forms a narrow strand or plug where the outlet of the follicles will appear. As we follow the further development of the young hair follicles, which are now attached to the junction epithelium (fig. 9, *h*), it will be seen that when they reach this stage of differentiation, just described for follicles on the epidermal surface, the expansion and separation of the lids will have brought their points of attachment to the free margins of the lids (figs. 12 and 13). So that when their hair shafts begin to form and to grow out, they will find a ready passage to the exterior. The junction epithelium is slightly increased in its longest diameter, now measuring 270 to 288 $\mu$ , while the shortest distance between the two lids is practically the same as at the three-day stage, viz., 40 $\mu$ .

*Five-day rat*

With the continued growth of the lid margins a slight change in the form of the junction epithelium is now seen. In addition to the deepening groove on the outer surface there is now dis-

tinctly seen a slight concavity of the conjunctival surface of the junction epithelium. The actual dimensions measured from the conjunctival concavity to the epidermal groove are, however, practically the same as at the four-day stage, viz., 270 to 288 $\mu$  by 40 $\mu$ . The conjunctival groove which is now first becoming definite is due to the expansion of the substance of the lid, while the junction epithelium remains stationary. This difference in the rate of growth is entirely responsible for the concavity on the conjunctival surface of the junction epithelium, and is partly responsible for the groove on the epidermal surface. In the latter case, the gradual keratinization of the epithelium is the other factor. This keratinization proceeds in the same manner as in ordinary surface epithelium by the preliminary formation of keratohyalin granules and final cornification of the cells, and has two loci. One is in the midline of the junction epithelium, forming a groove, and the other is in the mouths of the hair follicles attached to the junction epithelium when they are beginning to form hair shafts. The progress of the keratinization in these two loci combines to effect the major part of the disjunction process. The first, in the midline of the junction epithelium, is a continuous process, depending probably on the distance the surface epithelium is from the source of nourishment derived from the vessels of the tunica propria, and the second, in the marginal hair follicles, is associated with their arriving at a certain definite stage in differentiation. The resultant effect—the splitting of the epithelium—appears to be due to the same keratinization process manifesting itself jointly in these two situations. The time relations of the process in the two situations are closely interrelated, with the canalization process in the hair follicles usually slightly in advance. As a marginal hair follicle approaches the condition where it is about to become canalized, its point of attachment to the junction epithelium is found near the outer margin of the lid. In consequence, the line of canalization of the epithelium is usually through the outermost part of the junction epithelium and often ending in the outer groove. The presence of these little canals in the outer part of the junction epithelium seems to lead the

way for the deepening of the outer groove, and they serve as numerous little independent centers from which the cornification process extends into the adjoining junction epithelium.

### *Six-day rat*

At this stage the epithelial proliferation at the margins of the eyelids appears to have reached its maximum effect in the production of the junction epithelium. This is shown by the dimensions of the junction epithelium, which now measures  $288\mu \times 40\mu$ . From this time onward it will be seen that the actual measurement of the junction epithelium diminishes in its conjunctival-epidermal dimension, and, conversely, from the eighth day onward, the interpalpebral dimension increases. Henceforth, although epithelial proliferation and growth continue at the margins of the lids, no epithelium is added to the length of the midline of the junction epithelium. Since, however, the epidermal and follicular keratinization continues, the deepening of the epidermal groove continues, and the result is the beginning of the disjunction process.

### *Seven-day rat*

The conjunctival-epidermal length of the junction epithelium is now slightly diminished as compared with that found at six days, while the interpalpebral length remains the same. At the outer angle of the lids are long hair follicles, some containing distinct hair shafts. The tarsal glands have grown greatly in length and measure  $540\mu$ , but are still solid structures. But between the long well-developed follicles at the outer angle and the tarsal glands at the inner angle are again seen small follicles in early stages of development growing from the junction epithelium. This area of the lid is evidently still growing and differentiating and is still at an earlier stage of its developmental history than either the epidermal or conjunctival part of the lid. As the succeeding stages are studied, it will be seen that the disjunction process is concurrent with the further differentiation and growth of this part of the lid.

*Eight-day rat*

At this stage the process of separation of the lids may be regarded as definitely beginning, inasmuch as the form and measurements of the junction epithelium are distinctly altered (fig. 10). The shapes of the lids themselves are also modified at their margins. Under low-power magnification, it is seen that the width of the lid nearest the junction is distinctly thinner than the rest of the lid and that each lid tapers to its narrowest point at the place of junction with the opposing lid. The form of the junction epithelium is altered chiefly by reason of the deepening of the outer groove. The measurements are now  $216\mu \times 50\mu$ , and if these be compared with the preceding stage, it is found that the conjunctival-epidermal length has decreased and the interpalpebral distance has increased. Thus it is seen that while the epithelial proliferation at the margins of the lids continues and new cells are being constantly added, the effect now is to thicken the marginal epithelium on each lid, and not to increase the conjunctival-epidermal dimension, as measured in its midline. There are still a few marginal follicles attached to the junction epithelium which are solid cylinders of cells, and consequently have not advanced to the stage of canalization and do not contain hair shafts (fig. 10, *h*). The rest of the follicles at the margins which are attached to the free epithelium are conspicuously long structures, containing hair shafts which project beyond the epidermis. It is noticeable that the cells of the midline of the junction epithelium are very pale staining, as compared with cells in a similar position in the preceding stage, and this change in appearance indicates that they are about to become keratinized.

*Nine-day rat*

The length of the junction epithelium in the direction of the line of fission is only about half what it was at eight days, now measuring  $108\text{--}126\mu$  (fig. 11). This difference is principally by reason of the increased depth of the epidermal groove. The interpalpebral distance at the narrowest region of the epithe-

lium is practically the same as before,  $50\mu$ . The ducts of the tarsal glands show a well-defined lumen in the region near where the duct epithelium is continuous with the junction epithelium, but they have as yet no outlet to the outside. The alveolar out-pouchings of the glands are becoming well developed and their glandular cells begin to show their characteristic pale-staining mature appearance (fig. 11, t). By this time all the immature hair follicles which were seen at seven days attached to the junction epithelium now show hair shafts beginning to grow out. In the interval since seven days, however, the epidermal groove has also deepened so that the outlet of the hair follicles now open into it, and the hair shafts grow through the epithelium of the outer margin of the lid, not into the junction epithelium. There is, in a way, a process of eversion of the growing margin of each lid, which goes on all through the stage of separation of the lids.

#### *Ten-day rat*

Both the inner conjunctival furrow and the outer epidermal groove have become deepened, and in consequence the junction epithelium as measured between the two depressions is now reduced to a thickness of  $90\mu$ . In the epithelial cells lining the conjunctival furrow are now seen for the first time keratohyalin granules. These are in the form of small deep-staining particles, arranged in a linear fashion in the flattened, superficial, four or five rows of cells. The deepest layers of these cells at the bottom of the furrow come into a close relation with similar cells of the epidermal groove. The result is that keratohyalin granules are seen practically all through the midline of the junction epithelium. This represents a definite stage of advance in the disjunction process, inasmuch as it shows active progress in the keratinization of these cells. The interpalpebral distance is about  $75\mu$  at the narrowest region, and this is a considerable increase as compared with the nine-day measurement of  $50\mu$ . Although the lumina of the ducts of the tarsal glands are widely open even up to the marginal epithelium, no patent outlets were seen. The hair follicles at the margin of the lids have all attained



a mature condition, containing hair shafts and having outlets through the surface epithelium (fig. 12, *h*).

At this ten-day stage some variation in the condition of the junction epithelium was noted in animals from different litters. The description as given above was taken from the most advanced specimen and from a region of the lid about midway between the inner and outer canthi. In other specimens which were not quite so far advanced, the junction epithelium as measured from the inner to outer groove was greater in extent, and no keratohyalin granules were seen in the epithelial cells of the conjunctival furrow.

#### *Eleven-day rat*

Along the midline of the junction epithelium some of the cells, which contained the keratohyalin granules in the preceding stage, have now advanced to the stage of complete cornification. The connecting epithelial cells constitute a narrow eosin-staining band, bounded on each side by flattened cells, containing numerous keratohyalin granules. The granules are large and few in number within each of the cells of the one or two rows nearest the cornified zone and are smaller and more numerous in the cells of the succeeding two or three rows.

#### *Twelve-day rat*

The narrow keratinized band in the junction epithelium is becoming more distinct (fig. 13). This is the result of more of the cells containing keratohyalin granules becoming cornified. The dimension measured from conjunctival to epidermal side is lessened, and at the midpoint of the lid is about  $75\mu$ . Laterally to the middle region of the lid, this measurement is considerably greater. As the process of disjunction approaches an end, a slight but appreciable difference is seen in the appearance and dimensions of the keratinized band of epithelium in different parts of the same lid. At the middle of the eyelids the process of disjunction is more advanced, as compared with the condition laterally. This is to be correlated with the observation

that the first opening between the two palpebral margins will appear about the median portion of the lids. From here the opening process continues toward both the inner and outer canthus, usually reaching the former first. Outlets of some of the tarsal glands were open at this time, while in other cases a plug of cornified epithelium still occluded the mouths of the ducts.



Fig. A Photograph of section through margins of lids of albino rat thirteen days old, showing the lids connected by only cornified epithelium, and hence nearly ready to separate. Hair follicles occupy the entire thickness of the lids, except for a narrow zone on the conjunctival side where the tarsal glands (*t*) are situated. *x* marks position of outlet of duct of tarsal gland.  $\times 50$ .

#### *Thirteen-day rat*

The loosely arranged keratinized epithelium still connects the two lids (fig. A), but forms an extremely thin membrane. Its texture is less compact than at the preceding stage, its elements being curled and irregular and spread apart. The outlets of

the tarsal glands are distinctly open. The hairs and hair follicles of the marginal region of the lids are large and numerous. The whole appearance suggests the completed eyelids.

#### *Fourteen-day rat*

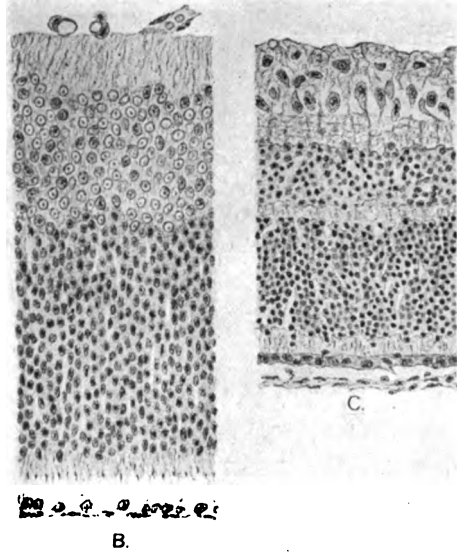
In some animals the final separation takes place on this day; in others, on the fifteenth day, and in a relatively small number the process is delayed until the sixteenth or seventeenth day. As mentioned above, the first opening usually appears in the middle region of the lids and proceeds in both directions, but usually reaches the inner canthus first. For some hours after the margins have separated at the inner canthus the lateral fourth of the lids may still be fused. The time necessary for complete opening after the first slit appears is often twelve hours. The lids on the two sides of the head of an animal do not necessarily separate at the same time. One may be entirely open before the first slit appears in the other. According to the observations of Dr. H. D. King, it often happens that in the same litter the eyelids of the females open before the eyelids of the males (Donaldson, '15). This circumstance is in line with the general precocity of the female at this period, as compared with the male. It sometimes happens that the eyelids are only partially separated for days after the usual time, and this gives them the appearance of being 'small-eyed' animals. In such cases, according to Dr. J. M. Stotsenburg, of The Wistar Institute, the separation may be easily completed by gentle pressure upon the lids with the tips of one's fingers.

#### *Structure of retina at time of fusion and of opening of lids*

At the time of fusion of the lids in the rat, on the eighteenth fetal day, the retina shows an early stage of development. The cells derived from the inner layer of the optic cup still form one continuous layer, but arranged in two zones (fig. B). This is also the condition present in the human retina at the time of fusion of the lids (Bach and Seefelder, '14). Of the two strata, the outer one is composed of closely arranged deep-staining cells,

and the inner one has its cells more palely staining and not so compactly arranged. No definitive visual cells are differentiated and no rods have yet developed.

During the period of the rat's development, when the lids are joined together, the retina undergoes its histogenesis. By the



**Figs. B and C** Two drawings to show comparison of states of development of retina at time of formation of lids and at time of their opening. In both the internal margin of the retina is shown toward the top of the page.  $\times 250$ .

**Fig. B** Retina of eighteen-day albino rat fetus at time of formation and fusion of lids, showing a very early stage of development and consisting principally of two broad zones of epithelial cells.

**Fig. C** Retina of fourteen-day albino rat at time of disjunction of lids, showing all the definitive layers of the functional retina. The proportions of the various layers, however, change with the further growth of the entire eyeball.

time of opening of the lid all the layers of the mature retina are clearly delimited (fig. C), though subsequent growth changes alter their relative proportions somewhat.

Thus it is seen that there is a close correlation between the time of opening of the lids and the arrival of the retina at a functionally active condition. Though the mechanism by which

the lids form and separate is entirely apart from the visual organ, yet the processes of development in the lids and in the retina are so coördinated that their definitive end-results are contemporaneous.

#### DISCUSSION OF OBSERVATIONS

In the albino rat two noticeable features in the formation of the eyelids are the late period in gestation at which the eyelids develop and the rapidity of their formation. The gestation period averages twenty-two days, and not until the eighteenth fetal day are the eyes covered by the lids (fig. 3). At the seventeenth fetal day the lids are represented only by ridges above and below the eyeball (fig. 2), but so rapidly does the process of formation proceed that in the space of a day the lids have grown across the eye, and have met and fused by their epithelial margins (fig. 4). As the lids approach one another in their growth, the epithelium of the margin of the developing lids is the site of especially active proliferation. In consequence of this, it grows out noticeably beyond the mesenchymatous portions of the lids (fig. 5), and first bridges the space between the approximating lids as a thin epithelial membrane. As the mesenchymatous portions of the lids continue to grow toward each other, this thin epithelial membrane is pressed together and portions of it are pressed aside as groups of epithelial cells (figs. 4 and 6), which are soon lost.

After fusion occurs, the junction epithelium continues to increase in the conjunctiva-skin dimension until about four days after birth. There is a temporary diminution in this measurement in the newborn, and this may be associated with the drying of the epidermis and the more rapid keratinization of the superficial epidermal layers of cells then beginning. After the fourth day the junction epithelium remains stationary until the sixth or seventh day. During this time the measurement is 270-288 $\mu$ . Then, due to the deepening of the epidermal groove, the length of this line of fusion becomes progressively less, and at the twelfth day is only 75 $\mu$ . On the thirteenth and fourteenth

days only cornified epithelium connects the two lids in the region midway between the inner and outer canthi.

The measurements of the junction epithelium in the interpalpebral direction at the various stages are in a general way the reverse of those in the conjunctiva-skin direction. Immediately after fusion at eighteen days, the interpalpebral measurements is  $63\mu$ , and as the mesenchymatous portions of the lids press together, this becomes less, so that at two days after birth it is only 30 to  $38\mu$ . It remains about the same until the seventh day, and then gradually increases, so that at ten days it is  $73\mu$ , and at twelve days  $90\mu$ . As the two lids continue to separate through the cornification of the epithelium in the midline of the junction epithelium, half of the epithelium belongs to each lid, constituting its surface layer.

Thus during the period of attachment of the lids, there are three stages, as indicated by the measurements of the junction epithelium: 1) Increase in length of line of fusion; 2) stationary attachment; 3) gradual separation. The first stage lasts until four days after birth, the second until the sixth or seventh day, and the third until final disjunction ensues at fourteen to seventeen days.

The period of attachment of the lids is related in time with especially two other developmental processes, the maturing a) of the skin and b) of the retina.

Being specialized integumentary structures, the lids in their developmental history follow the same general course as the skin. Hair follicles are developed both from the epithelium of the skin and from the junction epithelium. By following the development of the hair follicles which grow from the latter situation, one gets a sure guide to the time when the separation process is going on. For, as has been shown already, by the time the hair shafts form in these follicles, the splitting of the junction epithelium has proceeded far enough for the hair shafts to grow out directly through the epidermis of the margin of the lid. It is found that new hair follicles continue to arise from the conjoined epithelium for several days after most of the follicles have begun in adjoining parts of lid and head. As a

result, one finds the hair shafts first showing in these youngest follicles several days later than in the follicles elsewhere. Now in the albino rat the ordinary hair follicles begin on the lids during the eighteenth, nineteenth, and twentieth days, and the largest of these first show shafts within at birth. These begin to protrude through the skin at two and three days, and by the end of a week after birth there is a complete covering of short hairs. In comparison with this, we find that hair follicles not yet showing hair shafts within are found in connection with the conjoined epithelium as late as eight days after birth. At nine days these, too, begin to show differentiation of their internal cells, and by twelve days the hair shafts begin to appear above the epidermal surface. At this period a narrow band of keratinized epithelium has developed in the midline of the junction epithelium, and the final disjunction of the lids soon after occurs by the splitting of this keratinized epithelium. So the time of eruption of the youngest hairs at the margins of the lids is closely connected with the time of keratinization of the middle of the last remaining junction epithelium, which is the prelude to final disjunction.

From this survey it is seen that the period of time from union to disjunction of the lids may be correlated with a fairly definite period of development of the general integument of the body. Thus, the time of formation of the lids is just prior to the beginning of the epithelial proliferations which develop into hair follicles of the ordinary small hairs of the skin. One has to make an exception of the special large types of hairs, such as the vibrissae of rodents and the hairs of the eyebrows, upper lip, and chin of man. For in both rats and rabbits the vibrissae follicles make their appearance just before the time of formation of the eyelids. The above general statement holds true for man also, for while we find that both the lids and the hair follicles of the ordinary lanugo have their beginning during the third month of fetal life, the former practically always antedate the latter. But an exception has to be made of the follicles of the larger types of hairs of the eyebrows, upper lip, and chin, which begin toward the end of the second month.

After the lids are fused they remain in this state until after the arrival of the skin at the stage of cornification of its superficial epithelial layers, the formation of the hair shafts, and their appearance above the surface of the skin. In the rat this is about seven days after birth and ends the stage of stationary attachment.

The final stage, that of gradual separation, is associated with the maturing of the hair follicles which have originated from the junction epithelium. When the hair shafts of those which are nearest to the tarsal glands have erupted at about 12 days, the midline of the persisting junction epithelium has become cornified, and often in two days more final separation occurs. The duration of this separation process in common with other developmental processes is widely different in different species. Thus, in the rat, where the animal attains a complete coat of hair at about a week after birth, the separation process is completed at the expiration of another week or ten days. But in man, where the lanugo appears during the fifth fetal month and completely invests the body during the sixth month, disjunction usually takes place at the end of the sixth (Contino, '07) or the beginning of the seventh fetal month (Ask, '08).

The period of attachment of the lids is related in time also with the maturing of the retina. Functionally, the lids are a protective part of the ocular apparatus, and their separation does not take place until the retina has passed through its histogenesis. Thus, in the rat, at the time of formation and fusion of the lids, the wall of the optic cup has a relatively simple structure, with its cells arranged in two broad bands. During the period of attachment of the lids differentiation proceeds, so that at the time of disjunction, at about fourteen days, all the layers of the mature retina are recognizable. The same holds true for man, where at the time of fusion of the lids, about seventy days (Contino, '07, and Ask, '08), the retina has a simple structure corresponding to that of the rat at the eighteenth fetal day (Bach and Seefelder, '14). Separation of the lids occurs at the end of the sixth month or beginning of the seventh, and by that time the retina has structurally all the elements and



layers of the mature retina. After this time there is continued growth of the eyeball which alters the proportions of the several layers, but one can at least say that the retina at the time of disjunction of the lids is histologically mature.

The chief single factor in the separation of the lids is the keratinization process which appears in the surface cells of the epidermal side of the junction epithelium, as in epidermal cells everywhere (figs. 7 and 8). For a time the process does not manifest itself more actively here than elsewhere (fig. 9), but at a certain period, which we may associate with a definite stage in the maturing of the hair follicles of the skin, this process continues to progress inward in the midline of the junction epithelium (figs. 10 to 13), until it reaches the conjunctival side.

The mechanism of cornification in the junction epithelium is probably comparable to that taking place elsewhere over the integument. The history of the junction epithelium is, however, somewhat different from that of the ordinary epidermis, inasmuch as for some days after the lids fuse it becomes progressively thinner in the interpalpebral direction, then has a stationary thickness until the seventh day, and finally gradually increases. It is only when the last stage is reached that keratinization proceeds rapidly in the epidermal groove. This accounts for the delay in the process within the junction epithelium as compared with the general surface epidermis: Connected with these circumstances, causing delay, is also the fact that the margins of the lids are in a younger developmental condition than the remainder of the lids. At least, one may infer this from the fact young stages of hair follicles are still seen there when the adjoining follicles of lids and general skin show well-developed shafts. The increase in thickness in the junction epithelium goes slowly, probably because of the concurrent expansion of the marginal area of the lids. There is rapid proliferation of the epithelium, but many of the new-formed cells are necessary to keep up with the expanding marginal surface of the lid, and are distributed laterally. This is the condition up to six or seven days, by which time most of the hair follicles of the adjoining part of the lid are mature and their

hair shafts are visible on the outside. As is well recognized, hair follicles are the seat of numerous mitoses, and they are evidently now able to help supply the necessary cells for expansion and growth, each in its own local area. At any rate, it is at this time that the junction epithelium begins to increase in its interpalpebral dimension, and it is apparently this increase in the mass of epithelial cells which removes the cells of the midline of the junction epithelium farther from the source of nourishment in the mesenchymatous portion of the lids, and so favors the progress of the keratinizing process.

The tarsal glands are also considered to take a part in the opening of the lids, but they are found to participate to a comparatively slight degree in the albino rat. These glands have their beginning in the twenty-first fetal day, as epithelial proliferations from the conjoined epithelium, near the inner angle of the eyelid (fig. 7, *t*). At birth they measure  $70\mu$  in length, and at seven days have increased to  $540\mu$ , but are still solid throughout. At nine days the ducts show a distinct lumen, but it is not until the twelfth day that some of the ducts are found to have a direct opening to the outside, while most of the duct mouths are still obstructed by plugs of cornified epithelium. It is just before this last stage that the changes in the developing duct outlet may play a part in aiding the separation of the lids. For at this time the terminal part of each duct where it is continuous with the junction epithelium is still a solid mass of cells, although the greater extent of the duct is canalized. In the cells lining the duct immediately adjoining the unopened part some keratinization is going on, and as this process extends out to meet the similarly changing cells of the junction epithelium, the cornification of the conjunctival side of the raphe is accelerated. Schweiger-Seidel ('66), who was one of the first to study the process of lid separation in man, emphasized the formation of secretion in the sebaceous and tarsal glands as assisting in the opening of the lids, but this does not appear so important in the rat.

It is seen by comparing figures 7, 11, and 13 that the points of attachment of the tarsal glands to the surface epithelium remain

constant at the inner angle of the lid margin. By this comparison one may judge that the inner groove in the junction epithelium, which is so marked at late stages (figs. 12 and 13), is mostly the result of the expansion of the lid itself.

It is true that there is some formation of keratohyalin granules in the superficial cells of the groove, but the changes do not usually go on to complete cornification before the epidermal keratinizing process reaches them, and so can help but little in the separation of the lids.

Previous investigations of the developing eyelids have been made principally on human material, and of these the most complete are the studies of Contino ('07) and of Ask ('08). These contain many references to previous literature, beginning with Donders ('58) who was apparently the first to picture a thin section across the fused lids of the human fetus.

The observations on the eyelids of mammals, aside from man, are comparatively few, and are for the most part incidental to other studies on the developing eye. Ewetsky ('79) found in fetuses of domestic cattle that the fusion of the lids took place in fetuses of 5.5 to 6 cm. long, and that they separated in fetuses of 40 to 47 cm. In his figures of the approximating lids he shows the epithelium on the margins of the lids, growing out as a ridge in advance of the mesenchymatous part of the lid, as is shown in our figure 5.

Seiler ('90), who studied the development of the conjunctival sac, also examined the eyelids of young puppies at one, four, eight, and nine days after birth, as well as Talpa embryos. In the puppies he observed the formation of a broad cornified cell plate between the lids, leading to their separation.

Nussbaum ('08), in the course of his résumé of the process in man, refers to having also examined mice of two stages—at two days and at ten days after birth. In the two-day animal the stratum corneum of the epidermis showed no depression over the junction epithelium, but at the ten-day stage the process of cornification had advanced into the lid fissure, forming a groove which was funnel shaped in cross-section.

Schweiger-Seidl's observations on the process of separation in human fetuses were long quoted, and the figure illustrating the fused lid margins, which was published in 1866, is still in use in text-books of embryology. He regarded as the chief agencies in the separation, 1) the keratinization within the hair follicles developing from the conjoined epithelium and, 2) the formation of spaces between the cells of the junction epithelium by the secretion from the sebaceous and tarsal glands.

Contino ('07) studied a larger series of human fetuses, and considered that the separation of the corners of the lid margins was due to the secretion from the sebaceous and tarsal glands and that the separation of the intermarginal epithelium was due to the cornification of the central layers of cells.

Ask ('08), in addition to human fetuses, examined also a series of kittens, of which, however, he gives no description or figures, but says he found the conditions in both practically the same. He places the cornification process in the junction epithelium as the most important factor in separation. Cornification also proceeds from the hair follicles into the junction epithelium, and, thirdly, there is an independent cornifying process in the posterior part of the junction epithelium.

In our study of a consecutive series of rats of known ages, both fetal and postnatal, we have made measurements of the junction epithelium, and thereby have been able to attempt an analysis of the growth stages of the junction epithelium, and we have also correlated the period of fusion of the lids with certain stages of development of the skin and hair and with the histogenesis of the retina.

#### SUMMARY

The time of formation and fusion of the eyelids in the albino rat is a definite one, occurring during the eighteenth day of fetal life; hence observation of the presence or absence of the fused lids in a fetal rat will determine whether the fetus is older or younger than eighteen days.

The formation of the lids in this animal is a relatively rapid process; at the seventeenth day of fetal life there are only slight

ridges indicating the beginning of the lids, but in the succeeding twenty-four hours the lids have grown over the eyeball and have fused by their epithelial margins.

As the lids approach each other the epithelium at the margin of each lid grows actively in advance of the mesenchymatous portion.

By measurements of the conjoined epithelium, three stages are observed during the period of attachment: 1) increase in length of fusion; 2) stationary attachment, and, 3) gradual separation. The first stage lasts until four days after birth, the second until the sixth or seventh day, and the third until final disjunction at fourteen to seventeen days.

The period from union to disjunction of the lids may be correlated with a fairly definite period of development of the skin and hair. Thus the time of formation of the lids is slightly in advance of the first appearance of hair follicles of the ordinary small hairs. Then the lids remain completely fused until after the arrival of the skin at the stage of cornification of its superficial epithelial layers, the formation of the hair shafts, and their appearance above the surface of the skin (about seven days after birth). Finally, the stage of gradual separation is associated with the maturing of the hair follicles, which have originated from the junction epithelium.

The chief factor in the splitting of the junction epithelium during stage 3 is the process of keratinization, which continues inward from the surface epidermal cells of the raphe until it reaches the conjunctival side of the junction epithelium. During stage 3 the interpalpebral dimension of this junction epithelium gradually increases, and it is apparently this increase in the mass of epithelial cells which brings the cells of the midline of the raphe farther and farther away from the source of nourishment, and so favors the progress of the keratinization process within them. The inception of increase in the interpalpebral dimension is correlated in time with the arrival of the skin of the lid at its mature condition, while the margins of the lid are still in a younger developmental condition. By the time the follicles of the latter have developed hair shafts, the keratiniza-

tion process has penetrated the entire thickness of the junction epithelium, and disjunction soon follows.

The separation is also aided by the process of keratinization manifesting itself in, 1) the developing hair follicles derived from the junction epithelium, and to a less degree, in, 2) the ducts of the tarsal glands, and, 3) the cells lining the conjunctival furrow, just prior to disjunction.

Functionally, the period of attachment of the lids is related to the maturing of the retina. At the time of formation and fusion of the lids the retina has a very simple structure, consisting principally of two broad bands of cells, while at the time of disjunction all the layers of the mature retina are recognizable.

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## PLATE 1

### EXPLANATION OF FIGURES

1 Seventeen-day albino rat fetus, showing eye not yet covered by lids. Orth's fixation; photographed in 80 per cent alcohol.  $\times 3$ .

2 Section across eye region of seventeen-day albino rat fetus, showing eyelids represented by ridges above and below. Wistar Institute collection, no. 15370.  $\times 30$ .

3 Eighteen-day albino rat fetus, showing eye covered by lids. Fixation in 10 per cent formaldehyde solution; photographed in same.  $\times 3$ .

4 Sections across eye region of eighteen-day albino rat fetus, showing lids covering eye and fused together by their epithelial margins. Wistar Institute Collection, no. 15371.  $\times 30$ .

5 Section across eye region of another eighteen-day albino rat fetus, showing lids not yet in continuity, but their epithelial margins extending out as narrow ridges and closely approximated. The upper lid is thicker and shorter, while the lower is thinner and longer.  $\times 30$ .





## PLATE 2

### EXPLANATION OF FIGURES

Drawings of series of sections across junction epithelium of eyelids of albino rats at various ages, from nineteen days fetal stage until twelve days after birth. In all, the skin surface of the lids is shown toward the top of the page.  $\times 160$ .

6 Nineteen-day fetus. Eyelids fused together by their epithelial margins; (*ep*) groups of epithelial cells, which formed the projecting marginal ridges of epithelium in the approximating lids (fig. 5), and which have been turned aside by the lids pressing close together; (*h*) beginning development of hair follicle at outer angle of lid.

7 Twenty-one-day fetus. Increased thickness of junction epithelium in conjunctiva-skin direction; hair follicles (*h*) developing at outer angle of lid and on skin surface; (*t*) epithelium at inner angle of lid beginning to proliferate to form tarsal gland.

8 At birth. Junction epithelium somewhat decreased in longest diameter; hair follicles (*h*) in different stages; (*k*) keratohyalin granules very distinct in cells at outer surface of junction epithelium.

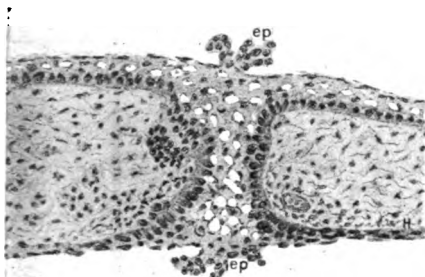
9 Four-day. Junction epithelium increased in length; (*h*) hair follicle buds beginning from middle of junction epithelium; a hair shaft is seen projecting through skin of lid; granules (*k*) of stratum granulosum very evident.

10 Eight-day. A distinct groove on outer surface where lids join; (*t*) cells of alveoli of tarsal glands are differentiating, but duct is still solid cord of cells; (*h*) young hair follicles developing from epithelium near outer angle of lid; (*s*) sebaceous glands of older hair follicles, which open on the skin surface.

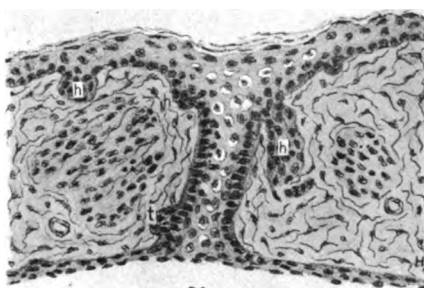
11 Nine-day. The epidermal interpalpebral groove has deepened; (*t*) ducts of the tarsal glands are canalized.

12 Ten-day. A groove has appeared also on conjunctival side of junction epithelium; (*h*) hair follicles and developing sebaceous glands; (*t*) alveoli of tarsal glands; keratohyalin granules are seen not only in cells of epidermal groove, but also in junction epithelium and in cells lining conjunctival groove.

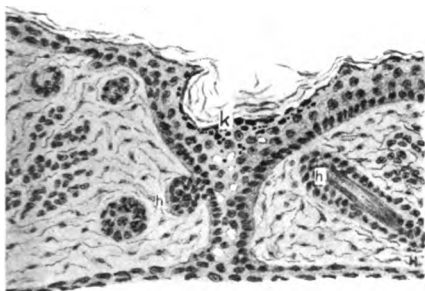
13 Twelve-day. Area of epithelial junction is much lessened and shows keratohyalin granules throughout; point of attachment of ducts of tarsal glands (*t*) is now opposite last remaining conjoined epithelium; owing to this diminution of the junction epithelium, the hair follicles which developed from it now open upon the epidermal surface of the lid.



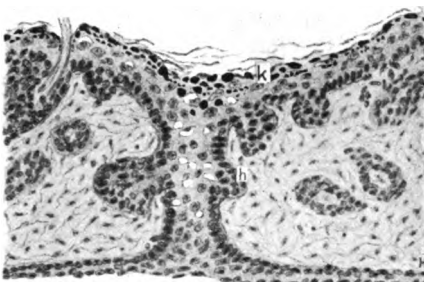
6  
19 DAY FETUS.



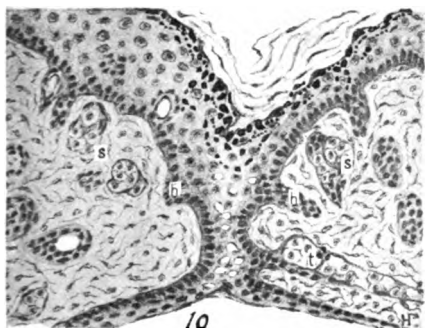
7  
21 DAY FETUS.



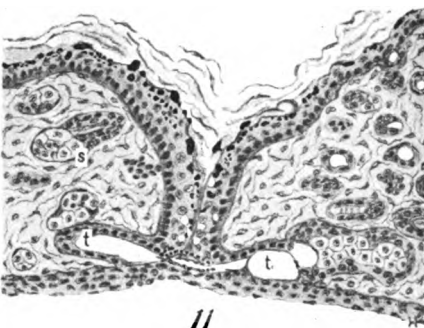
8  
AT BIRTH.



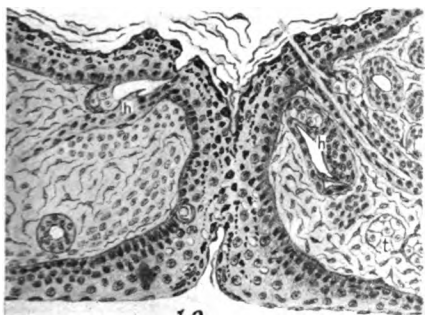
9  
4 DAY.



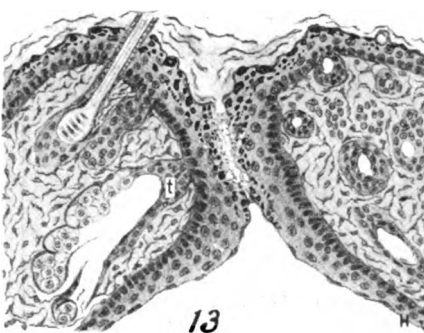
10  
8 DAY.



11  
9 DAY.



12  
10 DAY.



13  
12 DAY.

Resumen por el autor, Edward J. Stieglitz  
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Estudios histoquímicos sobre el mecanismo de la secreción renal.

El presente estudio del mecanismo de la función renal fué llevado a cabo siguiendo el curso de una sal de hierro (citrate amónico férrico), inyectada en las venas, a través del riñón, desde el plasma sanguíneo hasta la orina, comprobando la presencia del hierro con diversos intervalos mediante la reacción que produce el azul de Prusia. El autor ha comprobado que el hierro es segregado por los túbulos plegados, pero nunca pudo hallarle en los espacios glomerulares. Después de inyecciones múltiples el hierro se acumula en las células. Esta retención renal del hierro evita de un modo marcado y progresivo la secreción ulterior del hierro después de nuevas inyecciones y también reduce la producción renal de la fenosulfoneptale na.

Durante el periodo de eliminación del hierro disminuye el volumen de la orina, así como su peso específico, con una reducción en la cantidad de los carbonatos, demostrando esto una actividad inhibidora del hierro. Esta inhibición puede explicarse tomando como base el efecto del ión férrico sobre la capacidad de hidratación de los coloides del cuerpo, cuyo efecto evita el paso del disolvente, el agua, y por esta causa se disuelven, particularmente a consecuencia del hierro que se transforma en intracelular en las células secretoras. La inhibición o anti-diuresis no se debe a cambios en la presión sanguínea, a consecuencia de la inyección del citrate amónico férrico. La nefritis tubular experimental interfiere de modo muy marcado con la secreción del hierro. La concentración de la orina tiene lugar probablemente, principalmente por la secreción tubular más bien que por un proceso de reabsorción del agua y algunas sales.

Translation by José F. Nonidez  
Cornell Medical College, New York.

## HISTOCHEMICAL STUDIES ON THE MECHANISM OF RENAL SECRETION<sup>1</sup>

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*Hull Laboratory of Anatomy, University of Chicago*

SIX FIGURES AND ONE COLORED PLATE

The minute structure of the kidney required almost three hundred years of investigation before its intricate relations and delicate structure were clearly revealed. As yet the mechanism of urine formation by this highly complex biological apparatus is very poorly understood. A vast amount of work has been done in an effort to throw light upon the subject of renal function, but the results have been so varied, conflicting, and incomplete that we have not as yet arrived at a true understanding of the basic factors. The present investigation was undertaken to analyze the mechanism of renal secretion by means of histochemical studies. The work included: 1) A historical survey of the literature having direct bearing on the experimental studies and their interpretation. 2) The development of experimental methods. 3) A series of experiments tracing the course of a salt from the blood stream into the urine by microchemical methods. 4) A series of experiments dealing with secretory curves. 5) A series comparing the different behavior of two salts. 6) An investigation of functional changes in experimental nephritides. 7) A critical consideration of the results obtained. This report will first outline the main problem and then will discuss the various parts of the investigation in the order just given.

<sup>1</sup> This is a report of a series of investigations started in June, 1918, as the basis of a dissertation offered for the degree of Master of Science at the University of Chicago (1919), and continued through September, 1920, because of the interest of the work. The author wishes to express his deepest gratitude to Dr. R. R. Bensley for his generous and inspiring guidance and aid. Thanks for aid in some of the technical work are due to Mr. I. D. Siminson.

Bowman (1) in his memorable paper on the activity of the malpighian body in 1842, first conceived the idea that water and salts entered the urine from the blood through the squamous epithelium surrounding the glomerulus and that the organic substances of the urine were secreted by the convoluted tubules. This theory, greatly supported by the researches of Heidenhain (2, 3, 4) on the passage into the urine of sulphindigotate of soda, is in direct opposition to the conception postulated by Ludwig (5, 14) ('43), and now largely supported in a much altered form by Cushny. This theory in its original state assumed that water and salts, and, in fact, all the urinary constituents, passed through the malpighian body in the form of a dilute filtrate, which, as it passed down the uriniferous tubules, was concentrated by the reabsorption of water by the cells lining these tubules. At present, the theory, as modified and expanded by Cushny (6), includes the reabsorption of certain of the salt constituents which form the 'threshold bodies.' Of these bodies sodium chloride is perhaps the best example. It appears in the urine only when its concentration in the blood stream is above a specific level. In other words, there is a limit at which its elimination stops, the physiologic threshold. It is supposed by Cushny that this threshold is maintained to some degree by the reabsorptive activity of the cells of the convoluted tubules; he points out that such substances as belong to this group appear to be essentials in metabolic economy. Cushny (6, p. 50) conceives the two processes, that of glomerular filtration and tubular reabsorption, as being independent, but coordinated by the related common blood supply and by the rapidity of flow down the tubule. The last-named factor he deems especially important in connection with diuresis.

Küss (7) in 1881 suggested an amplification of the theory of Ludwig, namely, that the filtrate which came through the glomerulus was nothing less than blood serum in the form of a transudate, and that under normal conditions the serum albumin and globulin and other protein constituents were removed by the convoluted tubules. Despite the supporting observations of Posner (8) and Ribbert (9), this conception must be discarded,

as urine is by no means serum minus its albuminoid bodies (Sobieranski (10)).

The basic possibilities of a mechanism of renal secretion fall therefore under two main opposing views. The one places on the malpighian body the function of secreting or filtering by far the greater portion of the urine, which is later supposedly concentrated on its passage downward by reabsorption of the excess of water. On the other hand, the opposing view states that the concentration of the urine as it passes through the tubules is the result of secretion of salts and organic constituents into the lumen by the cells of the convoluted tubules. There is a third possibility to be considered, namely, that both mechanisms may play a rôle and are in a sense in equilibrium with each other.

The literature on experimental and anatomic evidence in connection with the mechanism of renal function is exceedingly voluminous. Most of the work that has been done can be classified under three general headings: Those studies dealing with the total renal function and the general effects of altered conditions; secondly, those pertaining to the function of the various units of the uriniferous apparatus and the localization of function, and, thirdly, the functional alterations occurring with distinct renal pathology. As examples of the first group of experiments there are the studies of salt concentration under varying conditions, the effects of diuretic stimulation (10), varying intraureteral pressures (11, 12), and variations in blood pressure and vascular supply (6, p. 99; 13). The localized studies on the function of the various elements of the uriniferous system concern us more in this paper. Among these investigations Nussbaum's justly famous work (15) of partially obliterating the circulation of the frog's kidney and noting the effect upon urine formation is perhaps the earliest which clearly recognizes the necessity for obtaining evidence of local function instead of studying the organ as a whole, as the function of the organ is nothing more than the composite function of its parts. Considerable work along similar, but modified, lines has been done since (16, 17). The work of Nussbaum and others is defective

inasmuch as the experimental method necessitates the production of a renal circulation nearly devoid of arterial blood and therefore introduces the unfortunate factors of asphyxia and malnutrition interfering with normal cellular activity. How essential these two factors are, Martin Fischer (18) has demonstrated.

A second type of investigation is that started by Heidenhain (2, 3, 4, 19) on the route of colloidal dyes in their elimination through the kidney. Gurwitch and many others have continued and elaborated such studies. Gurwitch (17, 20), working with 'indifferent' aniline dyes, agrees with Disse, v. d. Stricht, v. Gehuchten, and Nicolas in having demonstrated the presence of these dyes in the form of granules or masses contained in vacuoles which migrate toward and burst on the surface. Sauer (21), in opposition to such a conception, claims that the secretion is diffuse in the cells and is never in an aggregated form in cells or lumina of normal tissue. However, in such a discussion it would seem that the reaction of the tissue at fixation is a matter of considerable moment in determining the apparent physical nature of the secretion. Sobieranski (10) lays stress upon the inconclusiveness of their negative findings for the dye in the glomerular capsules, saying that such negative evidence is not proof, and offers three other explanations for such findings: 1) That there the dye may be too dilute to be detected microscopically; 2) that the dye may be reduced and decolorized at this point; 3) that the dye may pass through too rapidly to be detected morphologically. Goldman (22, 23) and many others, among them Evans (24), have devoted much investigation to the use of colloidal, so-called 'vital' and protoplasmic dyes. But all these experiments deal with the elimination of a foreign colloidal material which the kidney normally does not eliminate and which in its physical properties differs considerably from the normal urinary ingredients.

Some very excellent work has been done, however, using electrolytic salts instead of the dyes. Quinke (25) demonstrated iron in the uriniferous tubules and their epithelium, but does not localize this finding. Leschke (26) studied by microchemical methods the excretion of sodium chloride, urea, phosphates, uric acid, and other purines. He found that all these sub-

stances are detectable in quantity only in the convoluted tubules. According to his results, the glomeruli have no share in the excretion of these substances in any histochemically demonstrable quantity. Leschke's work was extended and confirmed by Oliver (27) in her work in connection with experimental uranium nephritis.

Pure cytologic studies of morphologic differentiation of the various parts of the uriniferous system belong in this group of investigations of local function, for morphologic specialization is always a criterion of specific function. This correlation is so universal that one can not discard such strictly anatomic data.

Lastly comes the group of investigations concerned with the altered function in conditions of renal pathology. To attempt to include any form of discussion of nephritis in this paper is impossible in view of the limited space, but as some of the most essential evidence of the true nature of renal secretion arises in the study of the experimental nephritides, a certain amount of discussion of this phase of the subject will be necessary. The work of Aschoff (28) and his student Suzuki (29) deserves especial comment, as they demonstrated the extreme specificity of various levels of the tubules in their reaction toward renal poisons. Their work will be taken up more in detail at a later point. The works of others, such as Potter and Bell (30), Pearce (31, 32, 33), Dickson (34), Lyon (35), Schneider (36), and Underhill, Wells and Goldschmidt (37), are all very valuable contributions to a clear understanding of renal activity. In connection with the results recorded in this paper these investigations will be discussed later.

The problem presents therefore the question: In which direction through the cells of the convoluted tubules are the urinary constituents passing; are they being secreted or reabsorbed? This was the original question upon which this work has been based, but the scope of the investigation broadened somewhat as it progressed. Wishing to avoid the use of colloidal dyes because their properties are foreign to the organism, as did Leschke (26) and Oliver (27), and desiring to use an electrolytic salt normally present in the body and yet detectable microchemically, we made iron the substance of choice.



## METHODS

Iron, for the purpose of tracing its course in the kidney, from the blood to the urine, has many advantages. In the first place, it forms ionized salts whose presence may be detected by very delicate but intense color-precipitation reactions leading to the formation of Prussian blue. This precipitation test is so delicate and so specific for iron that it may be used as a microchemical test in the finest histologic work. Furthermore, ferric salts, being electrolytes, should resemble in their excretion the behavior of other salts found in the urine. Iron is normally to be found in urine, and it increases greatly in those diseases involving the hematogenic apparatus. The normal presence of iron was first pointed out by Tiedemann and Gmelin in 1820 and afterward confirmed by a host of observers, although Becquerel, Herberger, Lehman, and Schlemmer denied its normal elimination via the urine because their methods were inadequate to detect the minute amounts. The accepted normal estimation for iron equals 1 mgm. per twenty-four hours for a human being (Neumann (38), Neumann and Mayer (39) and Kennerknecht (40)).

A further advantage in the use of salts of this element is that normal kidney does not contain any free (ionized) iron to cause a recognizable color reaction. This was determined by control experiments. Macallum (41) makes especial note of the absence of iron in renal cells. Therefore, any iron seen in the sections in the form of Prussian blue comes from the iron that has been administered to the animal. It was suggested that phosphates be used in place of the ferric compounds, but the objection that there were already phosphates present, which would interfere with the tests, ruled them out (42).

The use of iron in investigations of the mechanism of renal activity is not new. Glaevecke (43) as early as 1883 employed ferric citrate for such work. This salt was administered by subcutaneous injection. The first appearance of the iron in the urine, following such an injection, was one-half hour later. The iron concentration remained at its height from two to four hours following the injection, while the urine became negative only after a period of twenty-five hours. He also reports nega-

tive findings for iron in the glomeruli and Bowman's capsule, but records iron as present in the lumen of the convoluted and collecting tubules and in the cytoplasm of the cells of the convoluted tubules, along their free edge. As will be seen by the results described below, such findings are entirely in accord with those of our experiments. The chief, and serious objection to this work of Glaevecke lies in the method of administration of the salt. The subcutaneous injection does not allow of any accurate time relationship. An extra and unnecessary unknown factor is introduced in this way, namely, the rate of absorption of the salt into the blood stream and the equally indefinite period of administration to the blood. Throughout our experiments intravenous injection has been employed.

Kobert (44) also, in his study of the pharmacology of iron, has attempted an explanation of the mechanism of its elimination from the body, but his renal investigations have been so incomplete and inconclusive that the conclusions drawn are really not warranted. More will be said of this work at a later point. Quincke (25) also used iron.

The iron salt first employed in this investigation was the simple ferric citrate in the form of brown scales. But the salt is only sparingly soluble and required boiling before any clear solution could be obtained. Such a solution was always very dilute. As such a dilute solution requires the injection of large amounts of fluid to carry sufficient iron for histologic work, it would alter materially the activity of the kidney through its diuretic action. Therefore an attempt was made to use the compound salt, ferric ammonium citrate. The Kahlbaum preparation of the brown scales was first used. It was found to be freely soluble in water and in doses of 0.15 gram per kilo body weight was not noticeably toxic. But a series of experiments (K9, K11, K12, K13, and K14) of injection with larger doses (0.4 gram per kilo weight) demonstrated that the dose was invariably fatal in from one-half to nine hours. Repeated injections of the smaller dose of 0.2 gram per kilo weight were not fatal, but they led to albuminuria and the presence of casts in the urine. Therefore, this form of iron had to be discarded because of its toxic properties. We

were able, however, to procure a less toxic form of ferric ammonium citrate from Parke, Davis & Co., in the form of green scales. This salt caused no immediate toxic symptoms, even when as much as 0.8 gram was injected intravenously into a 2-kg. rabbit. In most of the subsequent experiments this form of ferric salt was used. For some time we were unable to get this product, and tried Merck's green salt, but found that it too was toxic in much the same manner as the brown scales. Several experiments were carried out to determine the chemical differences between these salts in an attempt to explain their marked difference in behavior in animal experimentation. It was at first suspected that the difference was due to some free ammonia in the brown salt because of the similarity of the symptoms to ammonia intoxication as recorded by Rachford and Crane (45). But the quantitative chemical analyses proved this supposition to be incorrect. Experiments K23, K44, K45, K48 are included in this series. The brown salt is described in the United States Dispensatory and the United States Pharmacopoeia as having about 16 per cent of iron. On analysis it was found that the green salt contained 16.4 per cent iron. The ammonia determination, by the Kjeldahl method showed the brown salt to contain 5.7 per cent  $\text{NH}_4$ , and the green 8.8 per cent. These percentages then show that the more toxic salt contains the smaller amount of ammonia. The composition of the two salts can be roughly calculated from these results.

Taking for the green salt the composition as being two ferric citrate molecules plus one of triammonium citrate, the calculated percentages are 7.3 per cent for ammonia and 15.2 per cent for iron. Therefore:

Calculated  $\text{NH}_4$  = 7.3 per cent.  
Analysed  $\text{NH}_4$  = 8.8 per cent.  
Calculated Fe = 15.2 per cent.  
Analysed Fe = 16.4 per cent.

This checks as closely as need be for the purity of a commercial products. In the same way, if we take two ferric citrate molecules, plus one of diammonium citrate, for the brown salt, the calculated percentages are: 5.03 per cent for  $\text{NH}_4$  and 15.65 per cent for iron. Therefore:

Calculated  $\text{NH}_4$  = 5.03 per cent.  
Analysed  $\text{NH}_4$  = 5.7 per cent.  
Calculated Fe = 15.65 per cent.  
Analysed Fe = 16.0 per cent (U. S. Dispensatory).

These later figures check even more closely, and therefore we conclude that the green salt is composed of two ferric citrate molecules combined with one of triammonium citrate, while the other contains a diammonium citrate. In the brown diammonium salt there is left one free carboxyl group which probably is the point of difference determining the toxicity. This view is further supported by the fact that on the addition of a slight amount of ammonium hydroxide to the toxic green salt, the toxicity is lost. Further, the green salt loses ammonia slowly, as determined by an examination of the air above the salt in the bottle containing it, and then shows toxic qualities. Therefore, we believe the green salt to be unstable, tending to lose ammonia and thus, through freeing a carboxyl group, becoming more toxic, and that the brown salt is merely a stage of more complete loss of the one molecule of ammonia. In experiments K73, K75, K76, K77, K78, K79, K81, and K82 all the animals died from the decomposed green salt. In all instances, as with the brown salt, there were marked nervous symptoms, convulsions, and respiratory distress. The convulsions appeared suggestive of vestibular disturbance, but no apparent change could be found in sections of the brain stem of K81, and the other tissues also seemed normal.

The fixation of the kidney tissues to be studied required a dual reaction. First, the tissues themselves must be fixed in a satisfactory manner and, secondly, the iron must be precipitated in the cell in the exact position that it had at the time of death. Any diffusion of the iron during the fixation would of course obscure any attempts to localize the iron. Aqueous reagents, such as plain Zenker's fluid, formol Zenker, formalin, and acetic acid Zenker, were first used, but although these yielded good results in tissue fixation, the iron was diffuse. Therefore 95 per cent alcohol was tried as a fixative, and it was found to yield the most satisfactory result, as diffusion of the salt was apparently eliminated entirely. There was some shrinkage due to the rapid dehydration, but control fixation with Zenker's fluid was always used to check the normal condition of the tissue.

Hall (46), in a series of experiments on the fixation of inorganic iron in tissues, found that pure alcohol fixes the iron in the same

position as was demonstrated by the ammonium-sulphide reaction in similar fresh tissues. The suggestion is made that ammonium sulphide ( $\text{NH}_4\text{SH}$ ) be added to the alcohol to insure the precipitation of the ferric salts. Tartakowsky (47) is in agreement with the findings of Hall and made extensive use of the sulphide alcohol as a reagent in fixation. In his estimation, formalin is as good a preserver of the original relations of the iron as pure alcohol. Abderhalden (48), on the other hand, states that it is his belief that alcohol destroys the localization of the iron. Such a conclusion has not been found valid in the present work. Macallum (41) states that ordinarily inorganic iron, as it is found in cells, is so slightly soluble in alcohol that the diffusion, if any, is not appreciable. He states that the forms of inorganic iron most frequent in the body are the phosphate, hydrate, and oxide. In his estimation, the use of ammonium sulphide in the alcohol increases the experimental error, as it may cause the liberation of organically combined iron already present (49).

The staining technique that was employed is very simple. Equal parts of freshly prepared 2 per cent potassium ferrocyanide and 2 per cent hydrochloric-acid solutions were used in the precipitation of the Prussian blue in the sections. A uniform counterstain was obtained with acid carmine, or better with the paler alum cochineal. The tissues were all imbedded in paraffin and sections cut with a rotary microtome. The routine technique employed called for two thicknesses of section: one of  $6\mu$  for the intracellular localization of the iron and the other for sections of  $24\mu$  for the anatomic localization of the salt.

One other method was used with considerable success in making the Prussian-blue preparations. A mixture of equal parts of green ferric ammonium citrate and sodium ferrocyanide was injected intravenously and the tissues then fixed in 5 per cent trichloroacetic acid. The hydrogen ion concentration of this solution is sufficiently high to cause the precipitation of the Prussian blue directly in the block of tissue and the reagent is at the same time a good protoplasmic fixative. Combinations of trichloroacetic acid with other reagents were not as successful,

although Carney's fluid with crystalline (anhydrous) acid, in place of the glacial acetic acid usually added, proved very satisfactory. The substitution of trichloroacetic acid in Bensley's A. O. B.<sup>2</sup> fixative proved a failure, as the efficiency in the preservation of the mitochondria was destroyed (47). In making the injections of the mixture of sodium ferrocyanide and the green ferric ammonium citrate, chemical equivalent amounts were used, with a dosage of the ferric salt equal to 0.2 gram per kilo body weight. By calculation it was determined that the ratio of equivalent weights was as 1 is to 1.06, and therefore in the work equal weights of the two salts were utilized.

Rabbits were the animals chiefly used, although some work was done with dogs, cats, and guinea-pigs. In all instances the salt was administered intravenously to insure more accurate time relations and eliminate the uncertainty of the time required for absorption. This forms a distinct improvement over the method of Gaevecke (40). A few experiments were also done with sodium ferrocyanide.

The investigations reported on in this paper may now be classified under several headings or series. The first of these (series I) was the original nucleus of the work and consisted in a series of preparations of the kidney from animals which had received intravenous injections of iron salts and had been killed at varying periods of time after the end of the injection. By such a series it was expected that one could note the direction of movement of the iron through the cells of the convoluted tubules, as the consecutive photographs of the cinema reveal motion. This series was carried out in full with the rabbit and shorter sets of experiments performed with the dog, cat, and guinea-pig.

Series II consists of a group of experiments pertaining to the retention of the iron by the organism and especially by the cells of the kidney and the physiologic effects of such retention. Series III includes a set of observations dealing further with iron

<sup>2</sup> Bensley's A. O. B. fluid consists of:

2 per cent osmic acid.....	6 cc.
2.5 per cent potassium dichromate.....	24 cc.
Glacial acetic acid.....	1 drop

elimination through a study of the secretory curves obtained by catheterization and of the effect of the iron injections upon the circulation. Series IV represent the investigations dealing with sodium ferrocyanide in contradistinction to the fate of ferric iron.

In the next group, series V, experimental nephritides, as produced by the almost specific renal poisons, tartrates, chromates, mercuric bichloride, uranium salts, and diphtheria toxin, are studied as to their effect upon the elimination of iron and it is attempted, through the localized pathology to support further the conception of localized, specialized, and specific secretion by the various units of the uriniferous tubule.

## RESULTS

### *Series I*

In this group of experiments iron salts, usually the green ferric ammonium citrate, were injected intravenously into rabbits and the animals killed at time intervals varying from two to five hundred and sixty (560) minutes later. The kidneys were immediately fixed and histologic preparations demonstrating the iron in the form of Prussian blue prepared.

The histologic localization of the iron in the kidney showed several constant characteristics, noted in every preparation. The first, most conspicuous and uniform factor was that the iron tended to be concentrated along the free border of the cells next to the lumen. In the earlier stages (two and three minutes) this was not so marked, but those preparations taken from experiments where the interval had been over thirty minutes showed the concentration quite conspicuously. The second common characteristic was that, in no case, if the technique of staining was carefully carried out, was there any evidence of iron in the connective tissue or any diffuse staining with the reagents. In regard to this notable absence of iron in the interstitial tissues a very interesting side issue developed, inasmuch as sodium ferrocyanide was demonstrated to be present (Prussian blue precipitate with trichloroacetic acid and ferric chloride or

the latter alone) in a diffuse form and in the connective tissues all over the body. This marked difference in behavior will be discussed under series V.

A most striking characteristic of the sections of series I was the entire absence of iron in the lumina of Bowman's capsules. In over three hundred fifty Prussian-blue preparations studied, iron was found in no case in the spaces of Bowman's capsules, although iron was often present in quite marked amounts in the capillaries of the glomerular tufts, and separated from the lumen only by the endothelium and the glomerular epithelium (43). On the other hand, iron was found in considerable amounts in the cells and lumina of the convoluted tubules.

In brief outline form, the histologic findings of iron in the above series is as follows:

*One-half minute (K88).* Female rabbit. Weight = 1550 grams. Injected 0.3 gram ferric citrate intravenously. The animal died immediately at the end of the injection, without a struggle, apparently as a result of embolism. Kidneys were fixed in:

95 per cent alcohol.

Zenker's solution.

Histologic findings: There was much iron in the blood-vessels and glomerular tufts, but none in any of the capsular spaces or in the convoluted or collecting tubules. With the Zenker control sections semi-scattered areas of round-cell infiltration and slight dilation of some of the medullary tubules were observed.

*Two minutes (K35).* Young male rabbit. Weight = 1450 grams. Injected 0.3 gram green ferric ammonium citrate plus 0.3 gram sodium ferrocyanide intravenously. Animal killed two minutes after the injection. Kidneys fixed in:

5 per cent trichloroacetic acid.

Carnoy's fluid with trichloroacetic acid.

Zenker's fluid with trichloroacetic acid.

Zenker's fluid.

95 per cent alcohol.

The bladder urine was negative for iron at death.

Histologic findings: In this experiment the major part of the iron was found in the blood-vessels, especially noticeable in the glomerular tufts of the malpighian bodies. There was no iron in the lumen of Bowman's capsule. There was some iron in the cytoplasm of the cells of the convoluted tubules, distributed in both borders of the cells and in every position in the cytoplasm in different cells. In the greater number of cases, however, the iron was in the portion of the cells away from the lumen. There was also some iron present in the thick limbs



of Henle's loop, where there was some slight diffuse cytoplasmic staining. In the medulla there was iron present in the lumina of the collecting tubules and blood-vessels.

*Two and one-half minutes (K42).* Adult female rabbit. Weight = 1970 grams. Injected 0.4 gram green ferric ammonium citrate intravenously. Animal killed two and one-half minutes later. Kidneys fixed in:

95 per cent alcohol.

Zenker's fluid.

The urine in the bladder at death was negative for iron.

Histologic findings: The preparations from this experiment gave nearly the same picture as those from experiment K35. Iron was found in considerable amount in the blood-vessels, but not conspicuously in the glomerular tufts as in the previous experiment. In the convoluted tubules, the iron was in every location in the cells in different individual tubules. Sometimes it was nearly confined to the border of the cells approximating the lumen, and in other examples it was to be found at the opposite periphery of the cell. There was some iron in the lumen of the convoluted tubules, in the form of distinct granules. In the medulla the iron was confined to the lumen of the collecting tubules and the blood-vessels.

*Three minutes (K38).* Adult female rabbit. Weight = 2300 grams. Injected 0.44 gram green ferric ammonium citrate intravenously. Animal killed three minutes after injection. Kidneys fixed in:

95 per cent alcohol.

Zenker's fluid.

The urine in the bladder at the time of death was negative for iron.

Histologic findings: In this specimen there was again considerable Prussian blue in the blood-vessels, but not so much in the glomerular tufts as before. In the case of the convoluted tubules, the iron was nearly entirely confined to the peripheral portion of the cytoplasm, away from the lumen. There was some iron in the lumen of the tubules and in the medullary rays and collecting tubules of the medulla.

*Ten minutes (K32).* Small male rabbit. Weight = 1620 grams. Injected 0.3 gram green ferric ammonium citrate, plus 0.3 gram sodium ferrocyanide intravenously. Animal killed ten minutes later. Kidneys fixed in:

95 per cent alcohol.

5 per cent trichloroacetic acid.

Zenker's fluid plus trichloroacetic acid.

Carnoy's fluid plus crystals of trichloroacetic acid.

The urine in the bladder at death was positive for iron.

Histologic findings: In both the 95 per cent alcohol and 5 per cent trichloroacetic acid specimens of this experiment nearly all the iron was present in the lumen of the collecting tubules and those of the medullary rays. There was some little iron to be seen in the blood-vessels. The iron in the convoluted tubules was present in the cytoplasm of the cells, especially near the free border of the cells at the

lumen. In some cases there was rather distinct Prussian blue staining of the intercellular membranes.

*Ten minutes (K39).* Adult female rabbit. Weight = 2350 grams. Injected 0.43 gram green ferric ammonium citrate intravenously. Animal killed ten minutes after the injection. Kidneys fixed in:

95 per cent alcohol.

Zenker's fluid.

The urine in the bladder at death contained iron.

**Histological findings:** In this experiment iron was present in considerable amounts in the blood-vessels. This was markedly noticeable in the glomerular tufts. There was very little iron present in the convoluted tubules and this was located primarily in the cytoplasm between the free border at the lumen and the nucleus. The lumina of the collecting tubules of the medulla and the pelvis of the ureter also contained Prussian blue in rather considerable amounts.

*Twelve minutes (K24).* Adult male rabbit. Weight = 1600 grams. Injected 0.3 gram green ferric ammonium citrate plus 0.3 gram sodium ferrocyanide intravenously. Animal killed twelve minutes later. Kidneys fixed in:

95 per cent alcohol.

5 per cent trichloroacetic acid.

Zenker's fluid.

The urine in the bladder at death contained iron.

**Histological findings:** Nearly all the iron in the preparations of this experiment was in the lumen of the collecting tubules of the medulla and in the medullary rays. Iron was also present in the lumen and cells of the convoluted tubules. In the cell the iron was scattered in the cytoplasm in the half of the cell toward the lumen of the tubule.

*Fifteen minutes (K25).* Adult male rabbit. Weight = 1970 grams. Injected 0.4 gram green ferric ammonium citrate intravenously. Animal killed fifteen minutes later. Kidneys fixed in:

Absolute alcohol.

Zenker's solution.

Urine in the bladder at death contained considerable iron.

**Histological findings:** There was a great deal of iron in the lumen of the collecting tubules. In the cortex the iron was found present in the medullary rays and diffusely in the cells of the convoluted tubules. There was also some Prussian blue in the lumen of the convoluted tubules.

*Twenty minutes (K28).* Adult male rabbit. Weight = 2300 grams. Injected 0.46 gram green ferric ammonium citrate plus 0.46 gram sodium ferrocyanide intravenously. Animal killed twenty minutes later. Kidneys fixed in:

95 per cent alcohol.

5 per cent trichloroacetic acid.

4 parts Zenker plus 1 part trichloroacetic acid.

Plain Zenker's solution.

Carnoy's fluid plus trichloroacetic acid.

The urine in the bladder at death was strongly positive for iron.

Histological findings (fig. 1): In the cortex of the kidney preparations of this experiment the Prussian blue was found to some extent in the lumina of the convoluted tubules and in a finely granular form in the cytoplasm of the cells, just under the brush border. There was also considerable iron in the lumina of the thick limbs of Henle's loops and also in the lumina of the medullary collecting tubules. None was found in the blood-vessels in the sections.

*Thirty minutes (K26).* Adult female rabbit. Weight = 1770 grams. Injected 0.4 gram green ferric ammonium citrate plus 0.4 gram sodium ferrocyanide. Animal killed thirty minutes later. Kidneys fixed in:

Absolute alcohol.

Carnoy's fluid plus trichloroacetic acid.

Zenker's fluid plus trichloroacetic acid.

5 per cent trichloroacetic acid.

The urine in the bladder at death was strongly positive for iron.

Histological findings: There was more iron to be seen in the cortex of the sections of this experiment than in the medulla; the greater portion of this cortical iron was in the form of finely granular clumps in the cytoplasm of the cells of the convoluted tubules, in the half toward the lumen, and in some cases apparently stuck to the brush border. Very little indeed was in any other position than this in the cells of the so-called secreting tubules. Prussian blue was also present in the lumen of collecting tubules, both of the medullary rays and of the medulla proper.

*Thirty-seven minutes (K10).* Large albino male rabbit. Injected 0.7 gram brown ferric ammonium citrate intravenously. Animal killed thirty-seven minutes later. Kidneys fixed in:

95 per cent alcohol.

Zenker's fluid.

The urine in the bladder was strongly positive for iron.

Histological findings: Practically every collecting tubule in the medulla of the sections of this experiment contained much iron in its lumen. The straight tubules of the medullary rays also gave a strong Prussian-blue reaction. It was noticeable that many of the convoluted tubules in the section were negative for iron, and that those which did contain iron varied greatly in the extent of their reaction. In some cases the cells were heavily laden at their peripheral border at the lumen, while others retained only a smaller amount. The localization of the iron in the cells was the same as described just above.

*Three hundred and thirty minutes (K13).* Medium-sized female 0.8 rabbit. Injected 0.8 gram brown ferric ammonium citrate intravenously. The animal showed toxic symptoms from the brown salt. Died five and one quarter hours later. Autopsied at once. Kidneys fixed in:

95 per cent alcohol.

Zenker's fluid.

The urine aspirated from the bladder was negative for the iron test. Histological findings: The medullary portion of this kidney was entirely negative for iron, as one would expect, as the urine of the animal had been negative before death occurred. But there was an appreciable amount of iron in the section. This was confined entirely to the cytoplasm of the cells of the convoluted tubules, at their border toward the lumen. Here again, there appeared to be iron right in the brush border of the cells in some cases, and it was noted that in some tubules the cells were heavily laden, while in others the cells did not contain any iron at all. This important point will be more fully discussed later in the paper.

*Five hundred and sixty minutes (K12).* Large albino male rabbit. Injected 0.8 gram filtered brown ferric ammonium citrate solution intravenously. Animal showed toxic symptoms. Died in nine hours and twenty minutes. Autopsied at once. Kidneys fixed in:

95 per cent alcohol.

Zenker's fluid.

The urine in the bladder was negative for iron.

. Histological findings: In the histologic preparations of this experiment the localization of the iron was exactly the same as noted in the previous experiment (K13). It was further noted that in both this period and the shorter interval of 330 minutes, those tubules which contained the iron in large amounts were distinctly grouped together and localized. The reasons for this will be discussed later.

The above-described type of experiment was repeated in shorter sets in three mammalian species other than the rabbit, namely, with the guinea-pig, cat, and dog. Essentially the same technique was employed except that a light ether anesthesia aided in making the intravenous injection. With each species three experiments, of typical time intervals of three, ten, and twenty minutes, were carried out. In the series with the cat the section fixed in Zenker's fluid and stained with hematoxylin and eosin in routine to check the normality of the tissue, showed all the animals to have been nephritic and therefore the results must be completely discarded. The same was unfortunately true of two of the dog experiments. The guinea-pig series, however, was normal throughout, as were all the rabbits used in the observations recorded above.

*Three minutes (K49).* Female guinea-pig. Weight = 740 grams. Injected 0.15 gram green ferric ammonium citrate into the femoral vein, exposed under light ether anesthesia. Animal killed by bleeding three minutes later. Kidneys fixed in:

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95 per cent alcohol.  
Zenker's solution.

Histological findings: Prussian blue was present in the cells of the convoluted tubules, and appeared in all parts of the cells in different instances. In many tubules the brush border was deep blue and the intercellular membranes demarcated. There was some iron in the blood-vessels (glomerular tufts), but none in any of the capsular spaces. There was only an occasional trace of blue in the lumina of the collecting tubules of the medulla.

*Ten minutes (K50).* Female guinea-pig. Weight = 720 grams. Procedure and dose identical with those of the preceding experiment, except that the animal was allowed to live ten minutes.

Histologic findings: In the medullary portion iron was present in the lumina of the collecting tubules. In the cortex the glomeruli and their capsular spaces were negative. In the lumen and also in the free border of the cells in occasional convoluted tubules granular iron was found. Those tubules containing the iron were grouped (K37), while many of the convoluted tubules showed no iron at all.

*Twenty minutes (K51).* Female guinea-pig. Weight = 655 grams. Procedures identical with those above. Dose = 0.13 gram (0.2 gram per kilo body weight, as was used as a standard), and the animal killed after twenty minutes.

Histologic findings: The medullary collecting tubules contained iron in their lumina. There was also some in the lumen and along the brush border of some of the convoluted tubules. The glomeruli and capsular spaces were negative. All in all, there was little iron in the tissue, less than with a rabbit.

Thus we have again, in a different species, results similar to those seen in the rabbit and differing only in the quantity of iron appearing, as the dosage is the same per kilo weight. There is no deviation from the trend of progress as more completely illustrated in the rabbit investigations.

Such a series of consecutive pictures, illustrating the trend of the iron to be from the periphery of the cells of the convoluted tubules toward their border approximating the lumen, and the presence of iron in the brush border only in the later periods of time, indicates very strongly that the passage of iron from the blood into the urine is by a mechanism of secretion through these cells, rather than as a filtrate from the glomerulus. In no instance was there a deviation from the general trend of the movement as witnessed by the localization of the Prussian blue. The negative evidence in regard to the finding of iron in the

glomerular capsule and the capsular space is of little value alone, because, as v. Sobieranski (10) and Brodie (14) have pointed out, the flow of fluid from the malpighian body may be very rapid indeed. Furthermore, negative evidence alone never carries conclusive weight. But, this fact, coupled with the evident localization of the intracellular iron, seems to warrant the conclusion that iron enters the urine through the secretion of the cells of the convoluted tubules. Glaevecke (43) records similar findings and arrives at the same conclusion.

### *Series II*

The last example of the above series in the rabbit showed that iron remained in the kidney for some time after the urine had become negative. This fact led to the work upon another series of experiments on the nature, both anatomic and physiologic, of this renal retention of iron. Aschoff (28) believes it important to differentiate the period of secretory activity and the 'Speicherungs phase.' The phase of secretion (for the dyes Aschoff used) reaches its height in an hour and then slowly falls, while the phase of storage only reaches its height after twelve to twenty-four hours. He concludes that storage or, as we have termed it, retention is an independent process from secretion. The results of our work, reported below, do not support such a contention.

The first fact sought for was the duration and behavior of the retention following a single injection of iron. In experiments K12 and K13, recorded above, we have seen that the cells of the convoluted tubules, especially along their brush border, contain the iron as long as 9.5 hours after the urine is negative with the very delicate Prussian-blue test. How long this iron would remain in the kidney following a single administration has not yet been exactly determined, but one experiment (K37) was carried out for the sake of an estimate, the animal being killed eighty-three hours after an injection of the usual dose of the green salt. It was about seventy-five hours after the urine had become free of iron. The sections displayed a little iron present

in the form of granular masses in the lumina of a few of the tubules of the medullary rays, somewhat after the nature of early casts. In a few cases small scattered granules of blue were to be seen in the cytoplasm of the cells of the convoluted tubules. The liver preparations were negative for iron, but the Kupffer cells were more numerous in the stellate form than normally found (51, 52). In the spleen, the macrophages contained much iron, but the splenic corpuscles were free thereof. In K52, where the animal was killed twenty-four hours after the injection of a corresponding dose, there was not apparently any more iron present. Therefore the rate of disappearance of this iron must be a slow one.

Any retention of the salt in the secretory cells would indicate that the cells are not able to pass on all the iron as they receive it, and that therefore, if the cells were made to receive still more iron, the retention should be accumulative. In experiment K30 three injections of the less toxic green ferric ammonium citrate were given in intervals of about thirty-six hours, thus permitting the urine to be free of iron for some twenty-four to thirty hours before the next injection. The animal was killed ninety-two hours after the last injection, or seventy-two hours after the urine had become negative. Histologically, a considerable retention of iron was demonstrated. The iron was confined to the cytoplasm of the cells of the convoluted tubules. It was again noted that certain tubules were entirely free, while others contained considerable quantities of Prussian blue. The phagocytic cells of Kupffer in the liver and the splenic macrophages also contained much of the reagent. Experiments K87, K88, K89, and K91 are of a similar nature. The routine Zenker control fixation revealed the kidneys of K87 and especially K88 to be pathologic, the former showing focal and diffuse areas of round-cell infiltration and the latter a distinct chronic fibrous interstitial nephritis. Therefore these two experiments must be discarded. In K89, in which the animal had received four injections of iron, the cells of the convoluted tubules were found to be diffusely blue, with a slight concentration of the color at the free margin of the cells, and the findings in K91, after the same

number of injections, were similar. In all cases considerably more iron was observed than following a single injection.

Having noted the evident accumulative nature of the iron retention in the secretory cells, it was believed that frequent long-continued, multiple injections might lead to a condition of block in the passage through the cell. Considering the iron to be passing by absorption from the urine to the blood, such a block should make the elimination of iron in the urine more complete and more rapid. But, on the other hand, if the block, either complete or partial, obstructs the passage of the iron through the cells from the blood to the urine, the elimination of iron should be both decreased and delayed. This question was investigated in a series of experiments on rabbits in which the animals were kept in metabolism cages and each sample of urine studied during a course of injections, and we were able to obtain a point where the block to iron elimination was very marked.

The first indication of an obstruction to the passage of iron was in the prolongation of the period of elimination. Following an intravenous injection of the iron salt, the normal period during which the urine remains positive is from four to six hours, but varies somewhat with each animal. In K30 (see above) the first period of iron elimination was approximately fifteen hours (the animal was not normal), the period following a second injection was forty-eight, and the third period forty hours. In K36, in which a normal male rabbit weighing 2350 grams was given nine intravenous injections over a period of twenty-eight days, the record of the tests for iron in the urine, taken every time a sample was voided, showed a constantly increasing period of elimination, until, following the last injection, it took 68.5 hours for the urine to become free of iron. This result might well be due to a block or impairment of activity on the part of the glomeruli; but if this were the case, the urine volume should show a progressive fall from day to day as the block increased. But such was not true, as the volume curve showed a very irregular secretion of urine, but without any progressive fall or rise in the amount. The suspected absence of any glomerular injury and the presence of an injury of, or an accumulation



of iron in, the convoluted tubules, was confirmed by the histologic study of this experiment. In the material fixed with Zenker's fluid, as control, the malpighian bodies were perfectly normal, but the cells of the convoluted tubules were small, surrounding enlarged lumina, and in some places there was desquamation. Hyaline casts were found in the collecting tubules of the medulla, but none was noted in the cortex. In the last days of the experiment casts were also noted in the urine. The cells of the convoluted tubules showed a more than normally granular cytoplasm, but the nuclei were apparently normal. With Bensley's aniline-acid fuchsin, methyl green mitochondria stain (50) the sections showed a loss of the mitochondrial content of the cells in the convoluted tubules.

In the tissue fixed in 95 per cent alcohol the kidney showed a great retention of iron. This was confined entirely to the cells of the convoluted tubules, and again showed the peculiar localization noted in the previous experiments (K12, K13, K30, K37, K89, and K91) (fig. 2). Some of the tubules were entirely free of iron and in others the cells were heavily laden. This same fact was observed in K102 (six injections) and K105 (five injections), although less iron was present. Those tubules containing the iron were distinctly grouped together. The tubules free of iron also showed the enlarged lumina and cells of decreased size described above. The significance of such a localization is not clear, but two possibilities are suggested. It may be an indication of a selective activity on the part of the different tubules, or on the other hand the localization may be due to some vascular change involving the afferent or even the efferent glomerular arteries, and thereby causing local constriction and an altered and reduced amount of blood to certain tubules. Kobert (41), in his paper on the pharmacology of manganese and iron, records the same type of findings for iron retained in the kidneys, but concludes that the difference in the tubules is due to a difference in the phases of activity in the various cells. Such a conclusion appears invalid in the light of the fact that this selective localization remains over a period of twenty-eight days.

Recognizing that it was quite possible that either the proximal or distal convoluted tubules alone retained (and therefore secreted) the iron, and being unable to differentiate them accurately in the alcohol-fixed preparations, two attempts (K72 and K89) were made to apply Huber's technique of tubule isolation to the iron-loaded ducts (53, 54). The animal of experiment K72 had received five injections. The Huber method of injection of concentrated hydrochloric acid into the renal artery was slightly altered by the addition of some sodium ferrocyanide to the acid to make possible the immediate precipitation of the Prussian blue. The kidneys became very dark blue, especially in the cortex. All the proximal convoluted tubules that could be identified with certainty contained iron, but not necessarily uniformly, for some cells were deep blue, while their immediate neighbors in the same tubule apparently contained no inorganic iron. In a similar preparation from K89, the animal of which had received four injections, the Huber micro-dissection showed many convoluted tubules containing no blue and small areas of blue in both the proximal and in some distal convoluted tubules. In K72 the findings in the distal tubules were not certain, as they were chiefly negative for iron. The glomeruli in both experiments were all uniformly devoid of blue. In other words, these experiments gave corroborative results which show that the iron is carried by individual cells in probably either the proximal or distal convoluted tubules (certainly in the former), but that these cells must be specialized and differentiated in some manner from their neighbors which do not retain and probably therefore do not secrete iron. In search of further evidence some kidney tissue containing iron (K96) was fixed in Bensley's A. O. B. reagent (50) for mitochondria and stained by Altmann's technique, but the iron was in a diffuse form, demonstrating the A. O. B. reagent as unsuitable for the fixation of iron. We believe that this subject of apparently highly localized special function warrants further investigation and that it is to be closely correlated with the studies of specific renal pathology of Aschoff (28) and Suzuki (29) (series V).

Besides prolonging the period of elimination of iron, the accumulative retention block decreases the quantity excreted. K72 was the first experiment which convincingly demonstrated this. Normally, the first sample of urine after an injection shows a very deep blue reaction with hydrochloric acid and ferrocyanide, a reaction read as + + +, followed by one of + + intensity, and then one of + about five hours after the injection. After the last administration (the fifth) in K72 only one sample showed iron and that was a single plus, 1.5 hours after the injection. Apparently very little iron was excreted, despite the fact that the usual dose was given intravenously. This decrease in the amount secreted was also noted in experiments K89, K91, K102, and somewhat in K105. No exact quantitative studies were made, but the colorimetric estimation is amply delicate to indicate the marked decrease. Together with the increased duration of the elimination, this fact is significant in yielding further support to the conception of secretion in contradistinction to reabsorption by the convoluted tubules.

Use was made in some of these investigations on the nature of iron retention of the phenolsulphonephthalein functional test of Rowntree and Geraghty (55, 56), which has been shown to be secreted by the convoluted tubules (55, 30, 57) and is extensively used clinically with considerable confidence and success and has proved far superior to other test substances, such as methylene blue (58), indigocarmine (3), rosaniline (59, 60, 61), potassium iodide (62, 63), and hemoglobin. At best, however, the introduction of such foreign substances for an estimation of functional renal efficiency is inconclusive, and a study of secretion of the normal constituents of the urine by the newer methods of biochemistry is perhaps a better indicator of the functional condition in disease. (Austin, (57)). In the rabbit a dose of 0.5 cc. or 0.03 gram of phenolsulphonephthalein given intravenously was used. The average normal excretion in eight tests was 61 per cent for the first, 8 per cent for the second, or 69 per cent for the two hours. In K72 the first normal reading was 33 per cent, 7.5 per cent, or 40.5 per cent for the total of two hours, while a test made after three injections of iron read 17 per cent, 4 per

cent and 21 per cent for the two hours, showing a striking decrease in the excretion. Not only does the accumulation of iron interfere with the further elimination of iron, but it greatly reduces the ability to pass the phenolsulphonephthalein, known to be secreted by the convoluted tubules.

The normal urine of rabbits is usually rich in carbonates; so rich, in fact, that it is generally turbid. This turbidity is lost and carbon dioxide generated by the addition of acid to the urine, which is alkaline to litmus. The carbonates are chiefly in the form of the calcium salt, for otherwise the turbidity would not exist. It was noted from the outset of the work that following an injection of iron the urine became clear for several samples. It also became brown (often quite dark) and gradually returned to its normal pale and cloudy condition as the concentration of iron diminished. The brown color was undoubtedly due to the presence of ferric hydroxide ( $\text{Fe}(\text{OH})_3$ ), as the urine was alkaline and the color was lost on the addition of acid. It is difficult, however, to explain the disappearance of the carbonate (iron containing urine did not give carbon dioxide with acid) during iron excretion. Whether calcium elimination is also inhibited at this time we cannot be certain, for it may have been present in other forms and no specific tests for calcium were made. According to Roehl (64), calcium is detected in the outer two thirds of the cells of the convoluted tubules following an intravenous injection of lime salts, and he concludes that these granules are in close relation to the excretion of the salt. This is a conclusion further supported by the accumulation of lime and the formation of soaps (Klotz (65)) when the kidney is damaged experimentally. Therefore it is quite possible that if calcium follows the same route as iron in its excretion, the presence of iron might interfere with and inhibit the passage of calcium, as it certainly does with the carbonate ions.

A further factor to be considered here, and also in connection with the other inhibitions resulting from the presence of iron, is its possible effect upon the physical condition of the cytoplasmic colloids as a trivalent cation. Loeb (66) has shown that bivalent cations, such as Mg, Ca, Sr, and Ba, do not cause excessive

swelling of colloid membranes as do the univalent ions and that this effect is primarily a surface one. It is his belief that any diffusion of electrolytes depends not only upon the osmotic pressure, but also upon the 'salt effect' upon the surface of the proteins on the membrane. Fischer (18, p. 51) states that iron (ferric) is the most effective electrolytic basic radical inhibiting the swelling of fibrin in water and acid. This is said to be equally true for other proteins. Calcium is considerably less effective in this connection, but among the acid radicals the citrate is the strongest. As both ferric and citrate ions are present during the period of secretion of such substances as phenolsulphonephthalein, carbonate, and probably calcium, they are directly associated with changes in the colloidal condition of the cytoplasm of the secretory cells. This effect is directly proportional to the concentration of the ions up to certain limits, an observation coinciding with the fact that with decreasing iron concentration the cloudy carbonates of calcium may gradually reappear in the urine before it is completely free of the ferric radical.

Assuming the Fischer theory of the physicochemical nature of water absorption, retention, and secretion by tissues on a basis of changes in the cell colloids (18, pp. 151 to 171), and realizing that the different parts of a single cell contain colloids of different natures, we find it is possible to explain the inequalities in water content (and similarly, salt content) of different parts of the same cell. An example of such inequalities is the accumulation of iron under the brush border in the first series. Fischer states (18, p. 165): "Concentration differences can be maintained in different parts of the same cell, between different cells or between cells and their surrounding media even in the absence of 'membranes' because of inequalities in distribution, determined by solubility, adsorption or chemical differences, or all three together." Meyer (67) and Overton (68) have pointed out that the rate of adsorption of substances by a cell is dependent upon the relative solubility in water and lipoids, or, in other words, upon its partition coefficient, and in the same way this controls the intracellular distribution. The adsorptive power

of charcoal is universally known, and colloids, also having tremendous surface areas, similarly are able to adsorb substances and so control their distribution. The adsorptive power of such colloids is greatly altered by environmental condition, as, for example, the hydrogen ion concentration, and therefore a given cell may adsorb, retain, or secrete depending upon the various influences at work upon it. Similarly, chemical combinations may bind a substance locally within a cell, but this is perhaps the least essential of the three factors.

Such a conception is entirely in agreement with that of cell polarity, both morphologic and physiologic. Direct evidence of such polar differentiation as seen in this work is the evident tendency for iron to accumulate under the brush border in the convoluted tubules. The idea of cell polarity is by no means a new one, and has long been recognized as an important factor in any epithelial secretory mechanism (69). The excellent work of Child (70) on metabolic gradients in unicellular and other low forms of animal life is an important contribution in this connection. In the higher forms Tashiro (71) demonstrated quantitatively different metabolic rates in different levels of a nerve fiber and changes in rate with the passage of an impulse from the higher to the lower level of activity. Alvarez (72, 73, 74) in a grosser way demonstrated a functional gradient of the alimentary tract which is independent of its anatomic units. And so it is not without precedent to suppose that within the individual cell the two poles may behave quite differently.

In the routine of the retention experiments the specific gravity of each sample was determined among the other tests. In thirteen retention experiments twenty-nine examples of the effect of iron secretion on the specific gravity of the urine were studied and in every instance the specific gravity was greatly lowered during the iron elimination. The average reading in fifty-seven observations on urine containing iron was 1.012 and the average for seventy-one readings of normal, iron-free urine, 1.0183. In many instances the difference was much more marked, as in K72: a reading of 1.006 when iron was present as contrasted to 1.018, 1.020, 1.022, when absent. This characteristic of lowered

specific gravity appearing when iron is present in the urine was such a constant one that it cannot be without considerable significance, particularly as it is correlated with the inhibitory or block effect of iron on the passage of phenolsulphonaphthalein, carbonates, probably calcium, and certainly of iron itself.

Were there a diuresis during the iron excretion one might assume the urine to be diluted because of the more rapid glomerular filtration of water, but no diuresis exists at this time. In fact, the findings are to the contrary, as there is a diminished elimination of water during iron excretion, as recorded and discussed under series III. Therefore, the urine cannot be diluted by the addition of an excess of water, and the only remaining explanation is that the presence of iron obstructs the secretion of an appreciable proportion of the urinary solids by the mechanism discussed above.

Therefore, we believe to have shown in this series of retention experiments that the cells of the convoluted tubules do not secrete all the iron that reaches them from the blood, but retain an appreciable quantity. This retention is accumulative with multiple injection of iron, and such an accumulation not only interferes with the secretion of iron as evidenced by a prolongation of the period of elimination and a diminution in the quantity excreted, but also inhibits the passage of other substances, and is therefore associated with a fall in the specific gravity of the urine. It is quite certain that this block or inhibition is essentially correlated with, and probably due to, an altered state of the cytoplasmic colloids brought about by the presence of the trivalent active ferric ions.

### *Series III*

A closer study of the changes in the urine and their relation to the interval after injection during the period of iron elimination promised to throw more light upon the mechanism of iron secretion by the kidney, and further investigations by means of catheterization experiments were carried out. The technique of these experiments was as follows: a rabbit, tied to the table

in the dorsal position, is catheterized and the bladder emptied; the usual dose (0.2 gram per kilo weight) of iron salt injected into the marginal ear vein and specimens of urine continuously collected, measured, and tested for iron at frequent intervals. The same quantity of urine and reagent were used in each test reaction, so that the colorimetric results could be read as trace, one, two, or three plus. The resultant data were charted.

The first of these experiments (K9) failed because the toxic brown ferric ammonium citrate was used and the animal died. In experiment K43 the following curve was obtained (chart 1):

This experiment was twice repeated (K46 and K60) with very similar results (chart 2).

All three of these curves agree so uniformly that the results may be considered as not due to chance or individual variation. The plateau of the iron secretion curve in reality probably should be a uniform rise and fall, but the triple plus readings were of such a dark blue that it was impossible to make higher readings. All the measurements of volume have been corrected, inasmuch as the intervals between taking samples are not uniform. The volume in cubic centimeters was divided by the number of minutes since taking the previous specimen and that figure multiplied by ten, putting all the reading upon an equivalent basis.

One of the striking characteristics of the iron-excretion curve is that the salt does not appear earlier than ten minutes after the end of the intravenous injection. In several other experiments of the type recorded under series I and II where the animals were kept in metabolism cages, and also in K9, this fact was also conspicuous when the animals incidentally urinated immediately or soon after the injection. In no such instance could a positive test for iron be obtained before ten minutes. A part of this latent period may be due to the time necessary for the urine to reach the bladder, for the delivery of the iron to the kidney is virtually immediate in an intravenous injection. This latent period alone contraindicates any passage of iron through the glomeruli, for other substances may appear in the urine in less than a minute. However, such evidence is only of value in the



light of the many other indications of secretion by the tubules. Furthermore, such curves correspond almost exactly with those found by Rowntree and Geraghty (55) for phenolsulphone-phthalein, known to be secreted by the tubules.

The most essential result of these experiments, however, is the decrease in the amount of water passed during the height of the iron elimination and the fact that as soon as there is a fall in the quantity of iron there is a corresponding rise in the volume excreted. This relation was also noted, but not so accurately or conspicuously, in many other experiments in which the urine

TABLE 1  
K43

TIME AFTER INJECTION	INTERVAL	VOLUME AS READ	CORRECTED VOLUME	IRON
		cc.		
8	8	0.5	0.6	—
23	15	0.5	0.3	+
28	5	0.5	1.0	+++
53	25	0.5	0.2	+++
113	60	1.5	0.25	+++
158	45	1.5	0.05	++
195	37	5.0	1.3	+
215	20	4.0	2.0	+
235	20	4.0	2.0	+
260	25	10.0	4.0	Trace
275	15	3.0	2.0	—
295	20	45.0	22.0	—

was obtained without catheterization, and therefore at more infrequent intervals. Indirectly, such a fall in the volume is further support for the view against the glomerular filtration of iron, as one should expect, an increase in the volume at the height of iron elimination, or an excessively active removal of water back from the uriniferous tubules into the blood would be required.

Associated with the antidiuresis is the finding that in several of the retention experiments (K72, K87, K89, K102, K105) a condition of ascites was noted at autopsy immediately after the animal was killed. This was especially marked in experi-

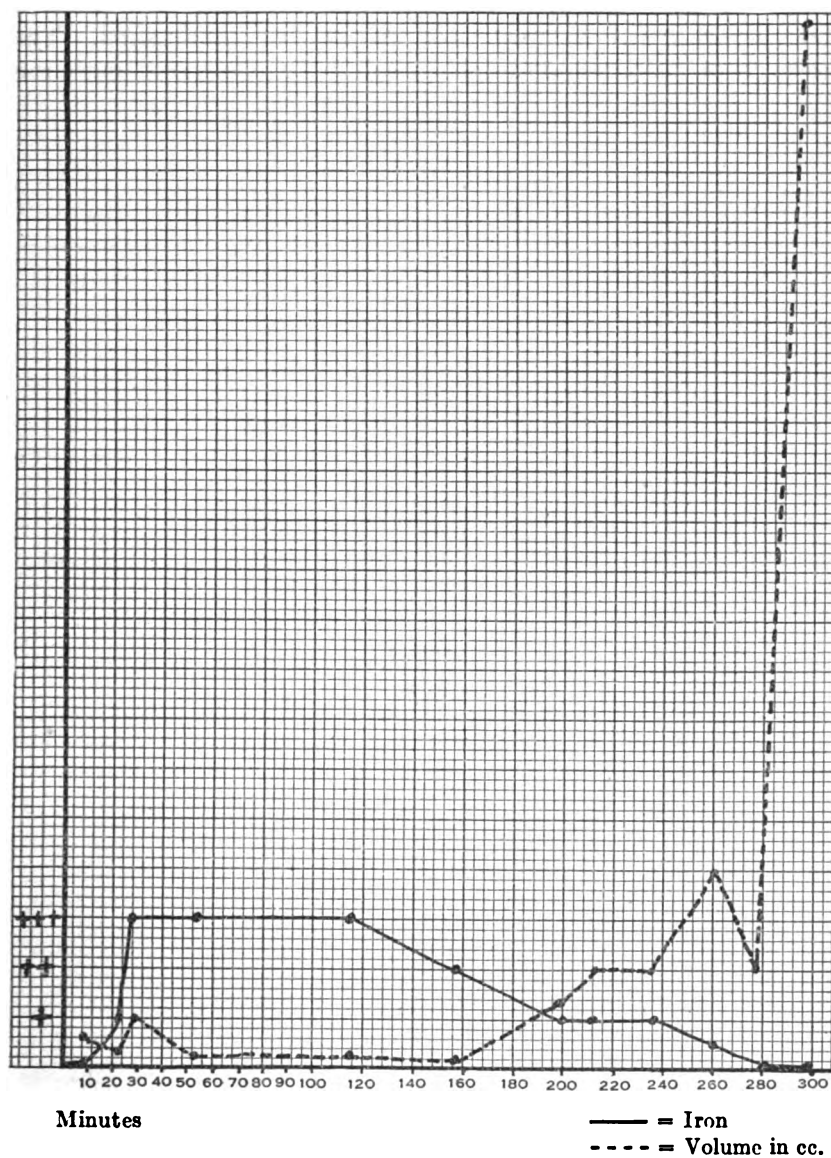


Chart 1 Catheter secretion curve of experiment K43

ments K102 and K105, in which the iron injections were pushed and the interval between administrations diminished. The presence of such ascites might possibly be correlated with the

TABLE 2

*K46*

TIME AFTER INJECTION	INTERVAL	VOLUME AS READ	CORRECTED VOLUME	IRON
		cc.		
6	6	0.5	0.8	—
10	4	1.5	1.2	+
20	10	0.5	0.5	+++
30	10	0.5	0.5	++++
40	10	0.5	0.5	++++
60	20	0.5	0.25	++++
100	40	2.0	0.5	++++
150	50	2.0	0.4	++
200	50	4.0	0.8	+
230	30	5.0	1.7	+
260	30	10.0	3.3	(+) Trace
290	30	40.0	13.0	—

TABLE 3

*K60*

TIME AFTER INJECTION	INTERVAL	VOLUME AS READ	CORRECTED VOLUME	IRON
		cc.		
3	3	1.0	3.3	—
5	2	1.0	5.0	—
8	3	0.25	0.8	—
11	3	0.25	0.8	+
23	12	1.0	0.83	+++
38	15	1.5	1.0	+++
63	25	1.0	0.6	+++
113	50	12.0	2.4	+++
183	70	7.0	1.0	+
253	70	30.0	4.3	+
288	35	12.0	3.4	—

effect of the ferric ion on the hydration of tissue colloids, particularly those of the renal cells. However, the data at hand in this connection are far too few to be of any conclusive value.

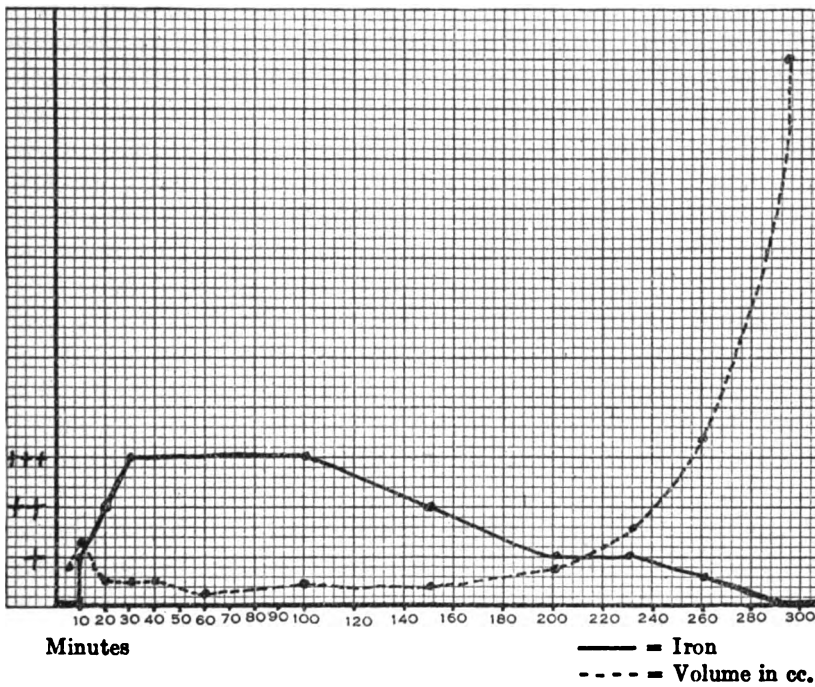


Chart 2 Catheter secretion curve of experiment K46, in which the green ferric ammonium citrate was used, as also in the other experiments of this type

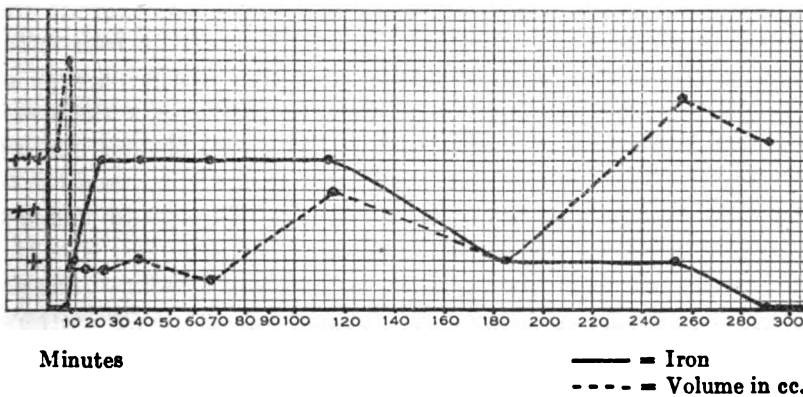


Chart 3 Catheter secretion curve, experiment K60

Realizing that this conspicuous antidiuresis or inhibition to water elimination might be due to some vasodilator, depressor effect of the ferric ammonium citrate interfering with the normal circulation to the kidney, we performed two experiments (K84 and K85) to ascertain this point. Dogs were used; the animals put under light ether anesthesia and cannular connections made from the carotid artery to a manometer set to record on a kymograph. The tracings obtained showed a prompt, very brief drop in the blood pressure of about 20 to 40 mm. when large doses were injected very quickly into the femoral vein. Both experiments gave the same results. This fall in pressure lasted only for a few seconds, never as long as a minute, and following that, the pressure remained at normal. Therefore it is impossible to ascribe the decrease in urine flow following iron injections to any depressor actions of the salt.

Another possibility which suggested itself in this connection was some change in the blood plasma, binding the 'free' water and manifesting itself by disturbance of the thermoregulatory processes and the production of fever, as recorded and discussed by Balcar, Sansum and Woodyatt (75). Therefore throughout the two above-described blood-pressure experiments the rectal temperature was taken at frequent intervals. However, no change in the slight and gradual fall of temperature, which invariably accompanies ether anesthesia, was discovered; the temperature curve fell uniformly, irrespective of the iron injections. Thus it is evident that the inhibition to the passage of water due to the iron salt is neither the result of a depressor action of the drug nor of changes in the blood plasma binding the 'free' water. Moreover, the presence of iron, tending to dehydrate rather than to hydrate the colloids, should increase rather than decrease the 'free' water.

However, Fischer (18, p. 295), in discussing saline diuresis, makes the following statement: "the saline diuretics are nothing but those salts which without being markedly poisonous are the most powerful dehydrants of the body colloids. They owe their action primarily not to any effect upon the kidney, but to an effect upon the body as a whole. By diffusing into the tissues

of the body they liberate water from them and their diuretic activity is but an expression of the amount of water they are thus able to liberate." Iron (18, p. 51) being the most active cation and the citrate radical the most powerful anion in dehydrating colloids, ferric ammonium citrate should be an efficient diuretic. How, then, can one conceive of the above results, showing considerable inhibition to water elimination, being compatible with Fischer's theory? They are, however, quite compatible therewith, as is clear from the following considerations.

In all three of the catheterization experiments there is a slight and very transient rise in the rate of flow at the very outset of the iron elimination. This may be due to any one of several factors; one of the simplest being the nervous and vasomotor stimulation occasioned by the fright and excitement of the animal during the injection. Such an explanation would not hold for the later, greater and permanent rise occurring with the decreasing concentration of iron, for at that time the animal is not disturbed. The brief rise might also be due to the increased blood volume due to the addition of the injection fluid to the circulation (dose usually dissolved in 10 cc. distilled water), but that is unlikely; or, lastly, to the fact that at that time, immediately after the intravenous injection of iron, the system is freely reacted upon by the ferric ion, the colloids altered so that they tend to lose water and then this excess of water must be eliminated by the kidneys.

Series I having shown how quickly the iron became localized intracellularly, the question arose as to how long the iron remained in the blood stream. Several experiments are included in this group, (K33, K38, K39, K40, K43), but in none was a conclusive result obtained, partly because the hemoglobin of the blood obscured the test by forming brown methemoglobin with the acid in the reaction. If iron were present it would be in very dilute solution and therefore a trace of blue would be lost in the deep brown. Furthermore, it is possible that the iron, when in the blood stream, is in combination with one or more of the serum proteins, and therefore insensitive to the test employed (49). From a study of the histologic preparations in series I

we have seen that the blood-vessel lumina give a positive Prussian-blue reaction up to a point of twelve minutes after the injection. Harvey and Bensley (76) record somewhat similar findings in their work on the gastric secretion of hydrochloric acid. Therefore, the duration of the free circulation of uncombined iron is probably quite brief, although it is quite possible that it circulates in a masked form, combined with serum colloids (proteins) with either chemical or physical bonds. Such iron would probably be inactive, as it is already bound to protein.

With the disappearance of the free and active iron from the circulation, the tendency for the body colloids to liberate water stops. Therefore, there is consequently no more stimulation to diuresis. Furthermore, the increasing accumulation of iron intracellularly in the walls of the convoluted tubules of the kidney begins to take effect. As we have just stated, we see that either the ferric or citrate ions alone, or more so together, reduce the tendency of colloids to adsorb water, and therefore the presence of either substance, but especially the ferric ion, in the cytoplasm of any secretory cell would tend to inhibit the passage into that cell of water or other substances, for the solutes cannot well enter if their solvent, water, is repelled.

Such is the mechanism we believe to be responsible for the initial, very transient diuresis and the later obvious inhibitory action of the iron to water secretion, and also to the solids of the urine, as evidenced by the decreased specific gravity during iron elimination. The apparent contradiction of Fischer's conclusions and results is due to two factors: 1) The fact that his injections of diuretic salines were slow and long continued, the body plasma therefore having a continuous supply of the saline, while in our experiments the administration of iron was by a single, rapid injection of the ferric salt, and, 2) that iron is conspicuously a salt which rapidly and thoroughly becomes localized intracellularly—a characteristic not described for the cations which Fischer worked with.

In one experiment (K67) similar to those showing the secretion curves of iron and water, 1 grain of caffeine sodiobenzoate was injected in the same solution with the ferric ammonium citrate and the following results obtained (table 4 and chart 4).

TABLE 4  
K67

TIME AFTER INJECTION	INTERVAL	VOLUME AS READ	CORRECTED VOLUME	IRON
		cc.		
2	11	3.3	3.0	+
4	2	1.0	5.0	++
7	3	0.8	2.7	+++
9	2	0.5	2.5	++++
11	2	0.5	2.5	++++
23	12	1.5	1.25	++++
49	26	1.4	0.5	++++
89	40	2.0	0.5	++
114	25	?	?	+
159	45	50.0	11.0	-

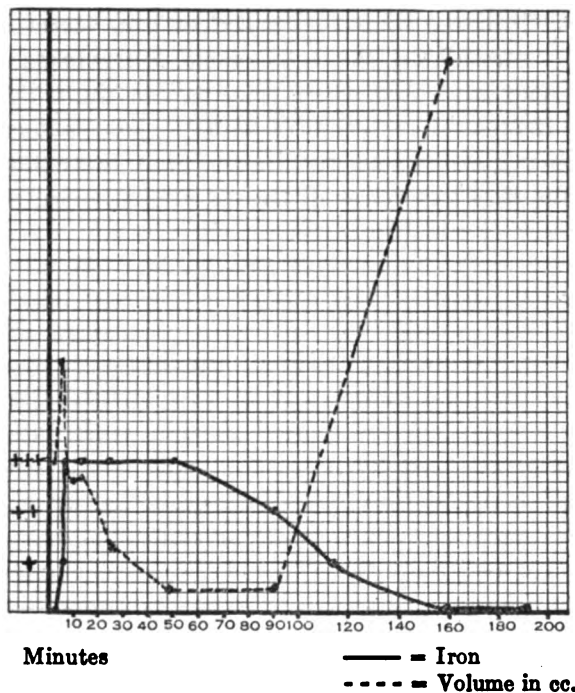


Chart 4 Secretion curve for experiment K67. With the ferric ammonium citrate injected was 1 grain of caffeine sodiobenzoate. Note the more rapid rate of secretion. Scale is the same as in the other figures



In comparing this curve with the results for iron injections without the addition of caffeine as a diuretic, one notes that the most striking feature is the much more prompt appearance of the iron in the urine and the greater brevity of the period of elimination, being 160 instead of 300 minutes. In all other respects the curves are similar. Corresponding with the shortened time of iron excretion, the rise in urine volume appears much earlier, but it is in the same relative position as before. The caffeine, although a diuretic known to increase the flow of urine, is unable to neutralize the inhibition of the iron, but as soon as that is removed a delayed diuresis occurs, as evidenced by the great volume of the last sample. It is not within the scope of this paper to enter into a detailed discussion of the pharmacodynamic action of caffeine and its related diuretics, but it is apparent, from the increased speed of iron secretion, that in some way it stimulated the cells of the convoluted tubules to greater activity and that there was a true diuresis, therefore, despite the decreased secretion of water. Iron is secreted more rapidly, water inhibited by the iron. That the caffeine can do this indicates that the route of the two substances, solvent and solute, is possibly not the same.

#### *Series IV*

The behavior of ferrocyanide in the body when introduced intravenously is quite different from that of the trivalent ferric ion. This was first demonstrated in this work by experiment K29 and later checked by a similar experiment, K49, both of the type of investigation described under series I. Prussian blue, precipitated by means of ferric chloride, was found in a diffuse form throughout the connective tissue, the renal pelvis and sinus and lymphatic spaces in the kidney sections and never in distinct granular form. Biberfeld (77, 78) and Basler (79) record finding the Berlin or Prussian blue in the interstitial tissues only and support their statements with figures. They are unable to draw any conclusions as to the pathway through the kidney, but find, as our experiments also show, that it appears

in the urine in abundance. Similar differences in behavior of the two compounds of iron in the animal body were recorded by Ranvier, as acknowledged by Miss Fitzgerald in her work on the secretion of hydrochloric acid by the gastric tubules (80). In testing for ferrocyanide in the fresh tissues at autopsy, we found that the salivary glands, stomach wall and gastric contents, the liver and subcutaneous as well as retroperitoneal connective tissue gave strong Prussian-blue reactions twenty minutes after the injection.

Such a diffuse and non-specific distribution is quite the opposite of that of the ferric iron, which appeared in a granular form, never in interstitial spaces and only intracellularly or in the blood-vessel lumina. In the introduction to this paper we made the statement that iron (ferric) was used because it is an ionizable salt giving a delicate microchemical color reaction. It is true that the salts employed are ionized when in aqueous solution, but when mixed with blood plasma the iron may become colloidal, possibly as ferric hydroxide, or be in loose physical or chemical combination with the serum proteins. That the latter alternative is the case is indicated by the particulate, granular nature of the intracellular Prussian blue, by the absence of a diffuse iron reaction in the interstitial tissues, and by the presence of the retained iron in the phagocytic cells of the liver and spleen, known to take up particulate matter (51, 52). Furthermore, the marked effect of the iron upon cytoplasmic physiology points toward some form of binding between the iron and the tissue colloids. Realizing this, it is not logical to generalize as to the mechanism of secretion of all electrolytes, and our conclusions are confined therefore to the *modus operandi* of iron elimination.

What, then, is the nature of the factors altering *in vivo* the distribution of the ferrocyanide? As it was suspected that it might be a purely physical factor, the diffusion rates into solidified gelatin were determined as follows: Solutions of ferri-ammonium citrate and sodium ferrocyanide were allowed to stand above gelatin in test-tubes for forty hours, and then, the solutions having been poured off, the gelatin was washed and treated

with acid and ferrocyanide and ferric chloride, respectively. Prussian blue was readily formed in both cylinders of gelatin, and the diffusion had proceeded to approximately the same depth in each instance. From the physical standpoint, therefore, there is no apparent cause for the difference in behavior discussed above.

From a chemical point of view, it is at once apparent that the ferrous iron has nothing to do with the actions of the salt, for it is firmly bound in the complex ferrocyanide radical. That this binding is firm and stable in the body is shown by the fact that the salt is non-toxic. Were the complex ion split, the free cyanide would be extremely poisonous. Neither can we lay to the sodium ion the responsibility for such behavior, and therefore we must assume that some characteristic of the ferrocyanide complex is the cause.

Thus, as it was thought that the cyanide radical might be, at least in part, responsible for this difference in behavior, potassium thiocyanate, which gives a very delicate, intense (red) color reaction with ferric chloride, was substituted (K62). But, to our surprise, no red color was detectable anywhere in the body ten minutes after an intravenous injection. In further tests (K64) it was found that the presence of either egg albumin or blood serum prevents the formation of the red  $\text{Fe}(\text{SCN})$ , and even decolorizes it after it has been formed. The action of the albumin was solely upon the thiocyanate, as the ferric chloride was shown to be free and uncombined by the addition of a little sodium ferrocyanide, which gave a liberal precipitate of Prussian blue. The presence of the sulphur atom in the cyanide radical made it so unstable that combination with protein resulted. However, such combination does not free a cyanide group, for the salt is non-toxic. Such a binding with proteins is not true with the ferrocyanide.

Two experiments, of the nature of those discussed under series III, were carried out, using a corresponding dose of sodium ferrocyanide in place of the green ferric ammonium citrate for injection. Charts and tables 5 and 6 give the results.

Two conspicuous differences between these results and those found for ferric iron stand out. The first of these is the much longer period of elimination (about 100 minutes, or one-third, longer), and the second the fact that the secretion of water is less inhibited, while the initial diuresis is quite marked. In connection with the prolongation of the period of elimination we can offer as explanation the fact that the ferrocyanide, being diffusely distributed through the tissue spaces all over the body, and probably retained there for some time, is more gradually delivered to the kidneys. The obvious tendency for the ferrocyanide salt to be scattered in the body, in contradistinction to the localization of the ferric ion, makes such an explanation conceivable, particularly as we have no clue to the exact and specific route of the ferrocyanide from the blood to the urine. From the histologic preparations (K29, K49) its entrance into the urinary passages appears quite incidental to its general diffusion, while the ferric iron is specifically focused and secreted.

Sollmann (81), in a discussion of diuresis, states that sodium ferrocyanide lowers the per cent of chlorides in the urine, but the absolute amount is increased, and concludes that the essential factor in the mechanism of chloride retention is the lowered quantity of the unbound sodium chloride in the serum. Sodium chloride is freed from its combination by  $\text{NO}_3$ ,  $\text{I}$ , and  $\text{SCN}$ , all of which raise the chloride concentration, but acetate, ferrocyanide, phosphate and sulphate ions, urea and glucose do not free the  $\text{NaCl}$  and therefore repress its excretion. In another paper (82) on comparative diuresis of different salines he states: "the diuretic effect is proportional to the dissociation and a specific diuretic action." That 'specific diuretic action' is, in all probability, the alteration of the hydration capacity of the body colloids.

Fischer (18, p. 51) includes in his series of anions inhibiting the hydration of colloids phosphate, sulphate, acetate, and sulphocyanate, all of which are also reported by Sollmann (81) as freeing sodium chloride from its combinations. Likewise, among the non-electrolytes, glucose decreases the hydration capacity, but urea increases it. Therefore, with the exception

TABLE 5  
K65

TIME AFTER INJECTION	INTERVAL	VOLUME AS RECORDED	CORRECTED VOLUME	IRON
		cc.		
2	8	1.5	1.97	Trace
4	2	1.5	7.5	+
6	2	1.0	5.0	+++
13	7	0.5	0.7	+++
23	10	1.0	1.0	+++
33	10	2.0	2.0	+++
43	10	1.0	1.0	+++
53	10	1.25	1.25	+++
68	15	2.0	1.3	+++
112	44	6.0	1.3	+++
151	39	1.5	0.4	+++
211	60	7.1	1.2	++
246	35	5.0	1.4	+
281	35	4.0	1.1	+
306	25	5.0	2.0	+
340	34	7.0	2.0	+
381	41	7.0	1.7	Trace
396	15	?	?	-

TABLE 6  
K104

TIME AFTER INJECTION	INTERVAL	VOLUME AS RECORDED	CORRECTED VOLUME	IRON
		cc.		
3	13	3.0	2.3	Trace
5	2	1.0	5.0	+
7	2	1.5	7.0	+++
20	13	1.75	1.34	+++
35	15	2.0	1.3	+++
50	15	1.25	0.8	+++
65	15	1.0	0.7	+++
70	5	0.75	1.5	+++
100	30	5.0	1.7	+++
130	30	4.0	1.3	+++
175	45	6.0	1.3	+++
205	30	4.5	1.5	++
235	30	6.0	2.0	+
265	30	3.5	1.1	+
295	30	5.0	1.7	+
355	60	9.0	1.5	=
385	30	4.0	1.1	Trace
405	20	?	?	-

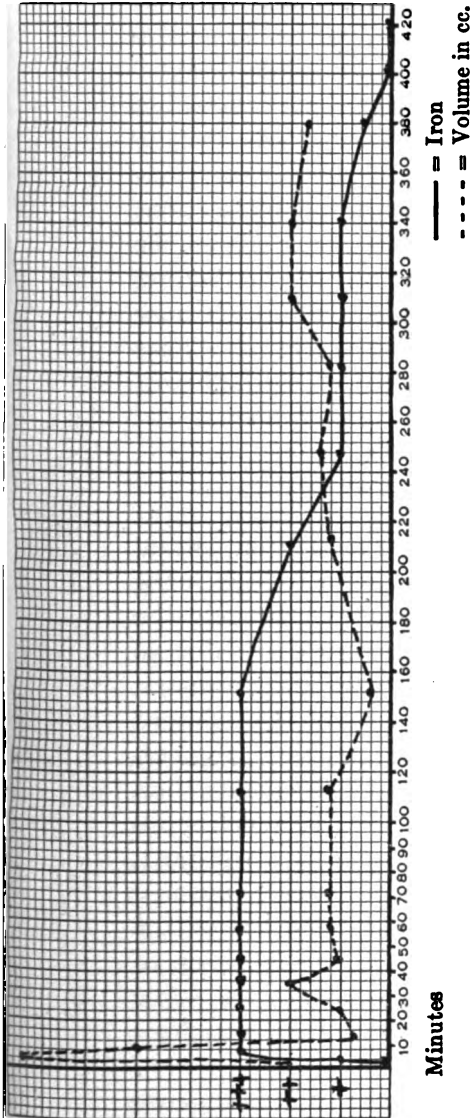


Chart 5 Secretion curve of experiment K65. Sodium ferrocyanide injected intravenously in place of ferric ammonium citrate. Note marked similarity and the distinct initial, transient diuresis

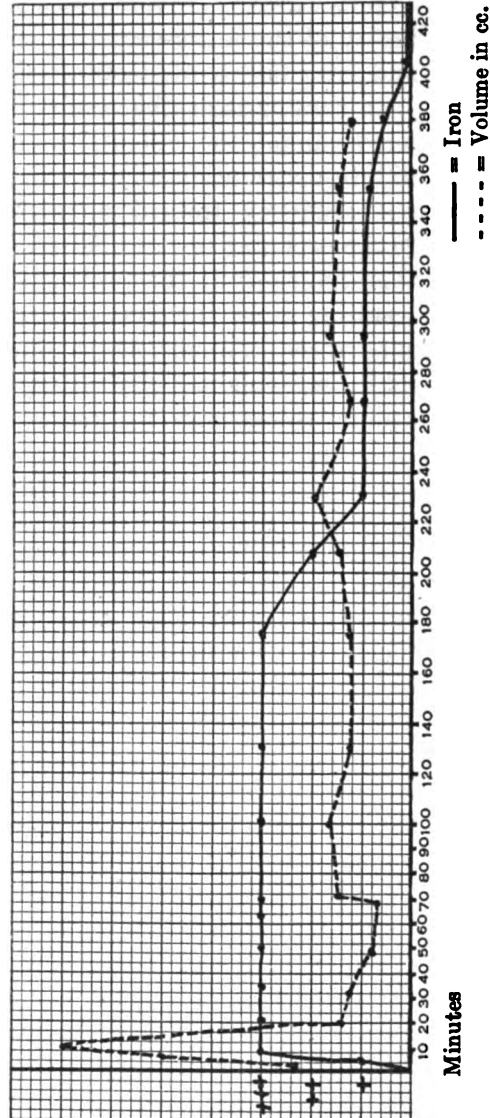


Chart 6 Secretion curve of experiment K104, a repetition of K65

of urea, all of Sollmann's series are inhibitors of colloidal adsorption of water. Therein probably lies the explanation of the similarity of the water-secretion curve, with its initial transient rise and later fall, to that obtained with the ferric iron injections.

In summarizing the results of these investigations concerning ferrocyanide, the essential points to be emphasized are: 1) The diffuse distribution of this salt in distinction from the conspicuous tendency toward intracellular localization of ferric iron; 2) the probable responsibility of the complex ferrocyanide ion for this diffusion; 3) the prolongation of the period of elimination, resulting from the first characteristic; 4) the relation of those substances stimulating sodium chloride excretion to those inhibiting colloidal hydration; 5) the peculiar susceptibility of sodium thiocyanate to become bound to albuminoid bodies.

#### *Series V*

The literature on nephritis is as voluminous as that on normal renal secretion. In this paper we will not attempt to broach the subject of pathologic renal function. However, through the means of experimentally produced renal lesions we are able to obtain a clearer insight into the normal physiologic processes. The great variation in susceptibility of the cells of the different units of the uriniferous tubules makes this possible. The collecting tubules are much more immune to injurious agents than the secreting tubules (35), as one would expect the much more highly specialized, differentiated cells to be far less resistant. Pearce (31) makes the following excellent generalizations: "The fundamental problem of experimental nephritis is the influence of the glomerulus as contrasted with the influence of the tubule." "The dual structure of the kidney is responsible for the difficulty which we have in interpreting the physiology as well as the pathology of this organ."

Clinically, the essential classification of nephritis is into acute and chronic forms. The latter are very difficult to produce experimentally, as the acute lesions tend to heal and return to normal, particularly if the cause is no longer active. Löhlein,

Leyden, Mann, and Councilman, all report large series of acute-nephritis cases which are followed by a quiescent period of several years and later the appearance of a chronic form of nephritis responsible for death. These, however, were of infective origin. Dickson (34) was able to reproduce chronic nephritis with prolonged administration of uranium nitrate.

However, from the point of view of the present investigations on the mechanism of renal secretion, the acute nephritides are the important class. They may be divided into those in which tubular damage plays the essentially important rôle and those in which the glomeruli are chiefly interfered with. Thus we speak of a 'tubular' and 'glomerular' or 'vascular' nephritis (83). These two types of lesions we may produce at will. However, the terms are merely relative, and in the glomerular nephritis there is undoubtedly some slight degree of tubular injury. The cause for the different localization of the deleterious effects of the various poisons used is that the lesions undoubtedly occur in that part of the uriniferous apparatus through which they are eliminated; the reaction of a cell to an intracellular poison is many times more violent than if the cell were merely bathed in an extremely dilute solution of the same poison.

Glomerular nephritis may be produced with arsenic, cantharidin, diphtheria toxin, or snake venom. Arsenic paralyzes the capillaries, causing marked functional changes with little or no anatomic evidence thereof. Catharidin causes a reaction both in the tuft and the capsular wall; from the latter there is considerable desquamation, and the convoluted tubules are also damaged. Diphtheria toxin causes the production of hyalin thrombi in the glomerular tuft and some necrosis there, with a mild degeneration noted in the convoluted tubules. The typical tubular nephritis is produced by uranium salts, mercuric bichloride, chromates, and tartrates, the latter in large doses. It is with the tubular form that we are concerned. Aschoff (28) and his student Suzuki (29) have carried the work on localization of the poisonous effects one step further, and claim that the convoluted tubule may be divided into three portions, each reacting quite specifically to certain poisons. This remarkable special-



ized susceptibility to such poisons strongly indicates a difference of function for the different divisions (upper, middle and lower) of the proximal convoluted tubule. For example, Aschoff states that uric acid is mainly secreted by the first and second divisions and that the glycogen in diabetes is chiefly stored and secreted by the last portion. It is on the basis of such remarkable localization of damage that the studies of this series were made, for with the tubules functionally destroyed, any alteration in secretion would indicate the route through the kidney of the substance excreted.

Of all the nephritis substances uranium is the most interesting. Not only is it the only poison with which chronic nephritis has been produced, but it is the only one which causes edema. The latter characteristic was first demonstrated by Richter (84, 85) and has been since confirmed by numerous investigators. The first microscopic evidence of uranium nephritis was recorded by Leconte in 1854 and the earliest careful observations reported by Chittenden and Lambert in 1889. Richter showed that there was more edema when salt and water were given than when water was given alone, and believed the chloride retention the essential feature of the edema. However, Georgapulos (86) found more water than chlorides retained while Schirokauer (87) reported a rise in the sodium chloride concentration of the tissue. Whether or no the salt retention is primary is still in doubt. Schneider (36), working with *Petromyzon fluviatilis*, was able to trace the source of the uranium through the cells of the convoluted tubules by precipitating a brown salt with potassium ferrocyanide, picric acid, and hydrochloric acid. Furthermore, in many of his specimens he found ferric iron, and reported similar pictures to those we have described under series I. In connection with uranium nephritis from the functional and regenerative points of view, the work of Oliver (27, 88) is of great value.

In the series of pathologic studies the experiments may be arranged into five groups—those dealing with the effects of uranium, tartrates, chromates, mercuric bichloride, and finally diphtheria toxin. These groups will be described separately.

*Group A (uranium nephritis).* That uranium salts attack especially the cells of the convoluted tubules we have just seen. The pathology is evidenced by granular and fatty degeneration and by necrosis of large groups of tubules (28). In our experiments (K95, K99, K100, K98) with uranium the Zenker-fixed material gave similar findings and the glomeruli were normal in appearance. The technique of all the experiments in this series followed a uniform course: An injection, either subcutaneous or intravenous, of a dose of poison big enough to cause a severe acute nephritis (0.02 gram uranium nitrate for a 3 kg. rabbit intravenously), followed twenty-four hours later by an intravenous injection of the iron salt. The animals were all kept in metabolism cages and the urine studied after each voiding. In both K95 and K100 the second injection was the green ferric ammonium citrate, and a great diminution in the amount secreted was noted. In K95 there was only one sample containing iron and that was a mere trace, while in K100 the iron-positive sample was reported as 'positive (faint).' Normally, the iron appears in liberal amount through two or more specimens of urine at intervals of one to two hours.

In K99 the animal received sodium ferrocyanide intravenously instead of the ferric salt. The one positive sample contained a considerable quantity of the salt. Following this voiding there was anuria until death, forty-four hours later. From the diffuse nature of the ferrocyanide distribution in the kidney, as elsewhere, one would expect little interference with its passage into the urine, whatever the pathology. But the distinctly localized passage of ferric iron should be greatly inhibited, as was found to be the case. Confirming Richter's observations (84, 85), we observed ascites at autopsy in K95, K99, and K100.

Thus we see a block to the secretion of iron in tubular nephritis, as produced by uranium. Similarly, there is a decrease in nitrogen elimination of from 9 to 14 per cent of normal (89) — a reaction the reverse of that found in glomerular nephritis, where the tubules must be hyperactive to compensate. In fact, in arsenic intoxication the urinary nitrogen is increased from 7 to 16 per cent, due to the increase in metabolic rate.

Siegel (90) confirmed these results. Green (91) also reported a fall (20) per cent) in urinary nitrogen in tubular nephritis when using chromates as the poison. Furthermore, Pearce (92) recorded the disappearance of a distinct depressor substance from the urine in tubular nephritis, but not following glomerular injury, and concluded the retention to be due to the loss of tubular function. Janeway corroborated this by observing a definite fall in blood pressure in acute renal insufficiency. Thus we see that tubular nephritis not only blocks the secretion of iron, but also that of other urinary constituents.

*Group B (tartrate nephritis).* That large doses of tartrates, either as the acid or in the form of Rochelle salts, when injected subcutaneously, produce a localized tubular nephritis is well known. Associated with this specific damage there is a diminution of urinary volume and a fall in the secretion of some of the solids (Underhill (93)). The alterations in renal function in tartrate nephritis were further studied by Underhill, Wells and Goldschmidt (37), and they conclude that the administration of alkalis modifies the severity of the reaction. It is a peculiarity of this type of experimental lesions that the severity of the reaction is frequently not in proportion to the dose, although large doses invariably cause a grave reaction. However, with the smaller doses the effects are present, but pass unnoticed when studied merely by routine pathologic methods, for in K22 we found that by the A. O. B. technique of Bensley (50) the mitochondria had been profoundly affected, although in routine material the cells looked perfectly normal.

From a functional point of view the reaction in tartrate nephritis is similar to that of uranium nephritis. With a relatively small dose (0.5 gram per 2 kg. body weight) the secretion of iron twenty-four hours later was not much interfered with (K20, K22), but with the severe reaction (2.0 grams subcutaneously) obtained in K94 only a very slight secretion of iron was recorded. One can conclude, therefore, that these results, associated with the findings in uranium nephritis, further support the conception of secretion of ferric iron by the cells of the convoluted tubules. Similar findings with phenolsulphone-

phthalein are recorded by Potter and Bell (30) in rabbits and Pearce and Ringer (94) in dogs.

*Group C (chromate nephritis).* Chromates, as do uranium and tartrates, cause damage to the convoluted tubules. The effects produced are practically the same as by uranium nitrate. In our experiments (K90 and K92) a granular degeneration with some vacuolization was virtually confined to the convoluted tubules, although there was some swelling of the cortical rays. Suzuki (26) believes that this poison affects only the proximal convoluted tubules.

The functional analysis of these experiments is greatly handicapped by the introduction of a complicating watery diarrhea from the subcutaneous administration of sodium chromate. The animal of experiment K90 died before iron was given. In K92 only one sample of urine positive for iron was obtained and that contained only a very slight amount. Apparently, therefore, the block to iron secretion with chromates is similar to that with uranium and tartrates, but this series cannot be used as giving conclusive results. Green (91) demonstrated a fall in urinary nitrogen in chromate nephritis corresponding to that of uranium poisoning (89, 90).

*Group D (sublimate nephritis).* Mercuric bichloride or corrosive sublimate is a well-known nephritic agent. It also affects the convoluted tubules (Wandervelde (95), Pearce (31)). Suzuki (29) states that the lower part of the proximal convoluted tubules is affected and Pearce (31) adds that there is also some damage to the ascending loop of Henle. There is rapid necrosis and cast formation with little previous change noticeable. Clinically, sublimate poisoning is characterized by a severe degenerative parenchymatous nephritis which locks the door to further elimination of the poison and therefore diuretic stimulation is of little avail in ridding the system of the bichloride.

In the first sublimate experiment the rabbit died three and three-quarters hours after the injection of iron, having had a convulsion similar to those arising spontaneously from the toxic, unsaturated form of ferric ammonium citrate. Usually these convulsions did not appear for from nine to thirty hours after

the injection. No iron passed through into the urine in this experiment (K97), and therefore we can conclude that the more rapid appearance of the convulsion is associated with the fact that the animal was unable to get rid of the ferric salt through the kidneys. In the other sublimate experiment carried out (K101) one specimen of urine contained a very little iron four hours after the injection of the ferric ammonium citrate. This was a great deal less than normally passes. Therefore, in association with the results from the other groups of this series, we have further proof that the route of iron secretion is through the cells of the convoluted tubules.

*Group E (diphtheria toxin nephritis).* Diphtheria toxin produces primarily a vascular or glomerular nephritis with hyalin thrombi in the glomerular capillaries and cortical arterioles, leucocytic infiltration and slight necrosis in the tufts, and a certain amount of degeneration in the convoluted tubules (31). Lyon (35) claims that diphtheria toxin produces almost wholly a glomerular nephritis. In K106 of our series besides the glomerular vacuolization the convoluted tubules appeared swollen and granular, but there was no necrosis thereof.

In experiments K106, K107, K108, we determined the maximal non-lethal dose (0.01 cc.). In two experiments (K109, K110) the injection of iron followed that of the toxin twenty-four hours later. In both cases there was a liberal secretion of iron, recorded as triple plus in K109 and triple plus one and one-half hours after the injection and plus four and a half hours after the injection in K110. Thus we see that the elimination of iron is very little interfered with in this form of vascular or glomerular nephritis.

From the above results we may conclude that the presence of a distinctly localized tubular nephritis greatly interferes with the secretion of iron, but a glomerular disease has relatively little effect. Thus this series of experiments offers further corroborative proof for the fact the iron (injected in the form of a true ionized salt) is specifically secreted by the cells of the convoluted tubules.

## SUMMARY

In summary of the results directly concerned with renal secretion recorded above, the following facts have been established:

1. There is a definite movement of iron from the periphery of the cells of the convoluted tubules to the lumina of the tubules, as evidenced by the Prussian-blue reaction.

*a.* No iron was at any time seen within the lumina of Bowman's capsules, even when it was present in the blood of the glomerular tuft.

*b.* The iron remains longest in the cells at their free periphery, just under, or in, the brush border during the period of iron elimination.

*c.* The ferric iron appears in a particulate form intracellularly; it is never found diffusely in the interstitial connective tissues.

*d.* Similar findings were observed in guinea-pigs as in rabbits.

2. Iron is retained in the cells of the convoluted tubules of the kidney after the actual secretion has ceased.

*a.* This retained iron remains present for at least eighty-three hours following a single injection, and probably remains longer, especially under the brush border.

*b.* The retained iron is confined to certain tubules and primarily to the proximal portion of the convoluted tubules; some cells contain much, while their immediate neighbors are free thereof.

*c.* The retention is accumulative with multiple injections.

*d.* The intracellular accumulation of iron produces an increasing block to its secretion into the urine; this block is evidenced by prolongation of the period of elimination and by diminution of the amount excreted.

*e.* The secretion of phenolsulphonephthalein is similarly interfered with by the iron retention in the convoluted tubule cells, and apparently also the elimination of calcium carbonate.

*f.* The intracellular presence of iron inhibits the excretion of water, evidenced in this series by the decreased volume during iron elimination and the appearance of ascites in retention animals.

*g.* During the period of iron elimination the specific gravity of the urine is distinctly lowered, demonstrating that less solids are being secreted at that time.

3. During the period of iron secretion there is an initial transient rise in the rate of water excretion, followed by a considerable inhibition which persists until the quantity of iron in the urine declines.

*a.* The initial diuresis is in accord with the physicochemical experiments of Fischer.

*b.* The inhibition to water secretion is due to the rapid and thorough intracellular localization of the ferric ions which are active in preventing colloid hydration.

*c.* The inhibition to water elimination cannot be explained on a basis of vascular depressor action of the drug or to the binding of 'free' water in the blood plasma.

*d.* Iron does not appear in the bladder urine earlier than ten minutes following intravenous injection of the salt.

*e.* With the simultaneous injection of caffeine similar results occur except that the initial and final diuresis are more marked and the duration of iron elimination is diminished.

4. The ferrocyanide complex ion is very diffusely distributed in the body.

*a.* The actions of this compound differ from those of ferric iron in the diffuse distribution, in the longer period of elimination and in the slighter effect upon the body colloids in connection with the adsorption of water by them.

*b.* The differences in behavior are due to the complex ferrocyanide ion.

5. The presence of a distinct acute local tubular nephritis greatly interfered with the secretion of iron.

*a.* Uranium nephritis, tartrate nephritis, chromate nephritis, and sublimate nephritis inhibit a block iron elimination.

*b.* Diphtheria toxin nephritis, which is primarily vascular (glomerular), produces very little interference with the elimination of iron.

## CONCLUSIONS

The mechanism of the renal secretion of iron, which we conceive to be the true one, on the basis of the above-recorded results, is one which permits of the actual secretion of iron from the blood into the urine by the cells of the convoluted tubules. This conception is therefore more in agreement with the original theory of Heidenhain than with the ideas of Ludwig and their modifications. That there is an actual biologic and vital secretion of the salt by the cells and not a mere mechanical filtration we believe to be clear not only from the above-recorded experimental results, but from the unanimously uniform results of other workers, among whom Oliver (27, 88), Quincke (25), Lescke (26), Gurwitsch (17), and Glaevecke (43) deserve especial note, as they worked with true salts. Although it is well known that the kidney functions primarily as an excretory organ, removing substances from the blood stream, but not elaborating new ones, its conspicuous greed for oxygen is equally renowned. This rapacious appetite for fresh arterial blood indicates a rapid metabolic activity such as would be required by secretory cells, but not by those functioning chiefly as membranes for mechanical filtration. The functional dynamics of secretory cells, in fact of all cells, undoubtedly rests upon a purely physical and chemical basis, but their structure and composition are so extremely complex that the explanation of their mechanics requires more elaborate study and understanding before we can speak of simple filtration, osmosis, or adsorption in connection with cells. Even the recent work on organic colloidal gels and sols must be interpreted as being fundamentally different from the existing conditions in cells where we have a highly complex and organized mixture of truly innumerable different colloids and solutes, all differing both in physical and chemical properties. To conclude that such an involved mixture behaves identically with a preparation of a single pure colloid is erroneous, but we may conclude that it will behave similarly.

The proximal portion of the convoluted tubule is the unit which is active in secreting iron. Whether or not the distal



portion is at all concerned therewith is not absolutely certain, but if it is, the secretion is minimal. We do not believe that this work has at all proved that there is no resorption of substances along the course of the uriniferous tubule, as postulated by Cushny, but it is clear that it is not the sole function to be ascribed to the tubules. The remarkable localization of function demonstrated in the process of iron secretion and similar specific localization of susceptibility to injurious agents (Aschoff and Suzuki) are facts of extraordinary interest, for they show us that two cells with identical appearance histologically may be endowed with different physiologic properties. Too much stress has been laid in the past upon mere apparent relationships and similarities and the conception of cellular and tissue specificity is a relatively new field, but one of vast importance. To the bacteriologists and serologists, not to the physiologists, belongs the credit for the earliest recognition of its importance. In this connection it is right to mention the illuminating work on chemical-tissue specificity done with the Abderhalden reaction (96). We cannot overemphasize the importance of the whole field of investigations dealing with such specificity.

Iron, probably through its active tendency to dehydrate colloids, produces significant functional changes in the renal cells. Not only is it accumulated, but its presence causes an inhibition of the secretion of further iron, of water, of carbonates, of phenolsulphonaphthalein, and of other urinary solutes (as evidenced by the lowered specific gravity). Thus we must consider that excessive iron retention prolonged over a considerable period must be injurious through preventing the normal elimination of the body's waste products. Therefore, although in the test-tube experiment iron should be an active diuretic, in the body it actually suppresses renal activity because of its conspicuous tendency to become concentrated intracellularly. Such prolonged retention damages the renal cells, and we believe that it is through such gradual, readily controlled and mild poisoning that we will eventually be able to arrive at conclusions regarding the mechanics of 'cloudy swelling.'

Furthermore, the ferric iron is active in inhibiting the excretion of water. That this is accomplished through its action upon the cytoplasmic colloids there is little doubt. Were the Cushny theory of renal secretion completely correct, the blockage to water in the cells of the convoluted tubules should greatly increase the volume passed, because the reabsorption of water would be checked. It is obvious that this is not actually the case. But it is equally clear that the urine is concentrated in its passage down the tubules, and we believe this to be accomplished by the addition of urinary solids, saline as well as nitrogenous, by secretion through the convoluted tubules. If there is any reabsorption of water, as is quite possible, there is little evidence that the convoluted tubules are involved. More likely is it to be associated with the loop of Henle.

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## **PLATE 1**

### **EXPLANATION OF FIGURES**

**1 Experiment K28. Twenty minutes following injection. High-power view of the cortex, showing the granular intracellular distribution of the Prussian blue under the brush border of the cells of the convoluted tubules. Note also the entire absence of iron in the glomerular capsule.**

**2 Experiment K36. One hundred and sixteen hours following injection. Low-power view of the cortex showing the patchy distribution of iron in certain convoluted tubules, typical of the retained iron.**



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2

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### Estudios sobre la dinámica de la histogénesis.

- IV. Tensión del crecimiento diferencial como un estímulo para la miogénesis del miembro.
- V. Compresión entre los centros de crecimiento acelerado del esqueleto segmentario como estímulo para la formación de las articulaciones.
- VI. Las resistencias al crecimiento esquelético como estímulo para la condrogénesis y osteogénesis.

De las pruebas presentadas en el trabajo adjunto se desprende que los diversos estados del desarrollo esquelético son los resultados de resistencias mecánicas al crecimiento. El miembro posterior presenta dos zonas principales que crecen longitudinalmente con diferente velocidad. El área esquelética central se acelera en su crecimiento, mientras que se retarda el crecimiento del mesenquima periférico. El alargamiento de los fascículos musculares en vías de desarrollo en el miembro, sigue la dirección del crecimiento longitudinal acelerado del segmento esquelético dominante ante próximo. Este alargamiento se produce por la tensión inducida en las masas premusculares por el segmento esquelético de crecimiento acelerado, rodeado por aquéllas. El cartílago y el hueso no son autodiferenciados, ni son tampoco productos autocristalizados, sino que son las respuestas celulares inmediatas a la intensidad variable de las presiones y tensiones producidas por resistencias (presión) que contrarrestan el crecimiento del esqueleto blastémico. Este último, en apariencia continuo, es en realidad segmentario y está compuesto de centros de crecimiento acelerado de acción opuesta. El contorno de la superficie opuesta que forma una articulación depende de la intensidad de la fuerza de crecimiento por milímetro cuadrado de sección transversa de los segmentos que forman una articulación, y también de las resistencias al crecimiento de los segmentos. Las resistencias, producidas por la interacción del crecimiento diferencial del miembro posterior, son activas y formativas en el origen y elaboración del esqueleto, articulaciones y musculatura.

## STUDIES IN THE DYNAMICS OF HISTOGENESIS

### IV. TENSION OF DIFFERENTIAL GROWTH AS A STIMULUS TO MYOGENESIS IN THE LIMB

### V. COMPRESSION BETWEEN THE ACCELERATED GROWTH CENTERS OF THE SEGMENTAL SKELETON AS A STIMULUS TO JOINT FORMATION

### VI. RESISTANCES TO SKELETAL GROWTH AS STIMULI TO CHONDROGENESIS AND OSTEOGENESIS

EBEN J. CAREY

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#### NINE FIGURES

#### 1. INTRODUCTION

The writer has presented facts ('19-'20 a; '20 b) which prove that the elongation of the developing muscular fasciculi of the digestive tract is in the direction of a dominant growth mechanical-energy extrinsic to the zone of myogenesis. This elongation is dependent on the fact that the primitive gut presents two zones of differential rates of growth. The inner epithelial tube is accelerated in growth, as compared with the outer splanchnic mesenchymal tube, which is retarded in growth. The growth of the inner tube is after the mode of a left-handed spiral, and is at first relatively more rapid in diameter than in length. The inevitable mechanical resultant of this differential growth is a tension of the outer tube in the same direction as the growth of the inner one. The inner close spiral muscle coat of the colon, in embryos of 14-25 mm. in length, begins to form during this initial transversely accelerated growth of the epithelial tube.

This initial muscle coat reacts upon the epithelial tube, thus restricting growth in diameter. This resistance causes the inner



tube to grow more rapidly in a longitudinal direction than in a transverse. The force of the growth of the inner tube during the period of its rapid longitudinal extension, in embryos 30-50 mm. in length, exerts a longitudinal, spiral tension upon the outer tube which had been retarded in growth. Concomitantly with the epithelial tube's period of rapid growth in length, the outer elongated spiral muscle coat is being derived from the splanchnic mesoderm.

According to the evidence, muscle formation in the gut is not due to a self-differentiation, nor to a spontaneous self-elongation of the myoblast, but is a dependent modification of the mesenchyme, due to the tension elicited by an extrinsic growth force. In view of other evidence yet to be presented, the writer is confident that this is the fact as regards all musculature. As an example, we may cite the spiral direction of the cardiac fasciculi corresponding to the changes in the vortical tension caused by the helicoidal blood stream flowing through the embryonic heart. In regard to the lingual musculature, an extrinsic force is found in the accelerated growth of the entodermal epithelium of the tongue.

The facts of direct observation prove that the formation of muscle tissue is a function of its position. Muscle tissue is formed in situ and is dependent upon an optimum tension, elicited by a dominant zone of accelerated growth, forcing by traction a connected zone retarded in growth. The direction of the resultant muscular fasciculi serves as a criterion of the direction of the dominant accelerated force which exerted the tension of differential growth.

In view of the evidence supporting the conclusion, that muscles arise through traction exerted upon the mesenchyme by a force extrinsic to the zone of myogenesis, it is as logical to claim that they self-elongate as it is to assert that a rubber band can stretch itself or a balloon can self-dilate. In the latter cases extrinsic forces are implied. As regards muscle origin, these forces are elicited by extrinsic zones of accelerated growth, inevitably drawing by traction retarded zones of growth, this being due to their relative positions to each other.

The reason for incorporating the three headings, involving the development of the muscles, joints, and modifications of the segmental skeleton, in this paper is the fact that the dynamic view-point, as regards limb development, necessitates the consideration of concomitant changes, in order to present the sequence of phenomena and evaluate cause and effect. The cause of the fundamental idea, first expressed in this paper, having been overlooked by previous observers is the fact that each tissue has been studied intensively as an entity isolated from the organism as a whole. The entire field of embryology must be reworked from the dynamic view-point of interaction, before a clean-cut idea of the physicochemical endowments of the primordial germ cell may be distinguished from that which is the mechanical resultant of the interaction of differential growth forces.

The purpose of this paper is to present facts of direct observation which prove that the formation of the skeletal muscles of the hind limb of the pig is dependent upon a tension of the somatic mesenchyme elicited by a force extrinsic to the region of myogenesis. The hind limb, like the gut, possesses two zones growing longitudinally but at different rates. There is an inner blastemal skeleton of accelerated growth and an outer somatic mesenchyme of retarded growth syncytially continuous with the skeleton. By the tensional interaction of this differential growth of the limbs, the skeletal muscles arise.

## 2. DIRECT OBSERVATIONS ON THE EARLY DEVELOPMENT OF THE HIND LIMB OF THE PIG (*SUS SCROFA*)

In embryos 6 to 10 mm. in length (fig. 1) the hind limb is represented by a convex bud covered with ectoderm and filled with a mass of uniform cells manifesting no signs of differentiation. As development advances, in embryos 10 to 14 mm. in length, a rapid condensation of the blastemal skeleton, proximodistally is occurring *pari passu* with limb extension. The nuclei of the syncytial skeletal core undergo rapid mitotic division. The first increase in size of the blastema is relatively more rapid in width than in length (fig. 2 and table 2).

This apparently continuous blastemal skeleton is in reality segmental in nature (fig. 2). The segments appear progressively from the proximal to the distal end, much in the same manner as that found in the successive caudal formation of metameres in the chick embryo. In contradistinction to the idea that segmentation of the continuous skeleton does not appear until joint formation occurs, the evidence at hand proves that centers of accelerated growth, segmentally arranged in the apparently continuous blastemal skeleton are prior in time to the formation of joints. This observation confirms that made by Bardeen ('10) on human embryos as noted in the following statement: "These cartilaginous anlagen are embedded in a dense blastema which shows lighter areas in the vicinity of the future joints." The same fact is recorded by Schulin ('79).

With the relatively greater increase in width than in length of the incipient femoral blastemal center, the peripheral cells of this rapidly proliferating zone show signs of retarded growth. They become elongated in the direction of the radial force of growth, forming an encircling constricting periblastemal membrane. With the formation of this limiting membrane, more rapid growth in length subsequently ensues in embryos 14 to 25 mm. in length (figs. 3, 4, 5 and table 2). This is due to the shifting of the planes of mitosis from a parallel to a transverse position, as regards the long axis of the limb.

The significant feature of the relatively and absolutely greater femoral growth in length during this period is the concomitant elongation of the nuclei of the premuscle masses in the direction of the skeletal growth. There is a subsequent stretching of the cytoplasmic myofibrils in the same direction. This inception of muscle differentiation of the thigh is more than a mere coincidence. It is an effect, a mechanical cellular response to the traction or tension to which the syncytially continuous and peripheral mesenchyme is subjected during the rapid growth of the femur in length. It would be as impossible for the femur to extend rapidly in length without elongating the continuous surrounding mesenchyme as it would be to expand a rubber balloon without stretching the limiting membrane.

During this period of rapid interstitial growth in length of the femoral blastemal center of the segmental skeleton, resistances are met with, first, at the proximal end in the centers of opposed growth for the ilium, ischium, and pubis; next, at the distal end in the centers of opposed growth for the tibia. The less condensed region, between the centers opposed in growth, becomes more and more condensed until a compression of the blastemal cells occurs along the line of juncture of the centers opposed in growth. Certain observers consider the segmentation of the skeleton as taking place at this stage and view the compression line as representing the beginning of the hereditary joints. The appearance of the joints, however, is the mechanical result of the opposed growth of antecedent, contiguous accelerated growth segments, and not the spontaneous cause of the segmentation.

With the inception of the outlines of, first, the hip and, next, the knee-joints, the contour of each skeletal segment becomes more defined. During this period the blastemal cells toward the center of the femoral segment become chemically changed by the intercellular formation of a cartilage matrix. This gives each cell the clean-cut appearance of a cell membrane. At the same time the primordial femur is bent, with its convexity toward the quadriceps extensor muscle and the concavity toward the hamstrings. The resistance to interstitial growth in length of the femur, due to the opposed centers at the acetabulum and at the knee, together with muscular contractility now manifested in the inception of the normal rotation of the hind limb, causes this characteristic bend of the femoral rod.

The attention of the observer is directed especially to the significant fact, that all cellular differentiation and degeneration occur first on the convex aspect at the center of the bent beam (fig. 5, no. 4). The first change from the blastema is the transformation into cartilage. Soon a dense perichondrial strain fibrosis is formed, followed by the degeneration of the cartilage cells and a calcification of the cartilage matrix. These retrogressive changes are due to the constriction of the blood supply, readily proved by injecting the embryos with india ink while

the heart is beating. All capillaries are then found peripherad to the perichondrium.

The last change in the above sequence of events on the convex aspect of the bent femur is the initial step in the formation of bone. The perichondrial fibrosis which effectively strangled the cartilage cells becomes modified into a periosteal membrane. Where the cartilage cells are degenerating a proliferation of the osteoblasts from the deep aspect of the periosteum is seen. These cells replace the dead cartilage cells. The osteoblasts form a matrix which is mechanically situated to serve effectively as a cellular reaction to the great strain to which the bent femoral beam is subjected. This appositional growth of bone serves to strengthen the femoral beam at its weakest part. The direct mechanical result of this cellular reaction is the progressive formation of a more stable base for the application of muscular forces.

The various steps in the increase of skeletal density, first, from the blastemal to the cartilage period, and, secondly, from the cartilage to the osseous period, in skeletal condensation is next to be considered simultaneously with those changes extrinsic to the zone of skeletal formation. During the early stages of development, the weight of the entire limb is supported by the femur acting like a cantilever beam. The weight of the limb rapidly increases (table 1 and chart A). In an 18-mm. pig embryo the femur constitutes one-fifth of the volume of the limb and supports a weight of 0.013 mg.; in a 38-mm. embryo the femur constitutes one-sixth the volume of the limb and supports a weight of 0.125 mg., whereas, at the 50-mm. stage of the developing embryo, the femur constitutes one-seventh of the volume of the limb and supports a weight of 0.25 gram. Later, at the 20-cm. stage, the femur constitutes only one-sixteenth of the volume of the limb, yet it supports the greatly increased weight of 30 grams. In addition to sustaining the above weight, the femur is opposed in growth by the accelerated growth centers located proximally and distally. Finally, as development continues, the resistance presented to longitudinal femoral growth by the contracting and elastic reacting muscula-

ture is an opposing factor to be considered as extrinsic pressures limiting the relative volume of the femur to the thigh.

With the rapid increase of limb weight and with increase of opposition to growth at the ends of the femur, together with the resistances manifested by muscular reaction, the density of the femur progressively increases (tables 2 and 3 and chart B).

TABLE 1

EMBRYO			HIND LIMB	
Length	Weight	Volume	Weight	Volume
cm.	grams	cc.	grams	cc.
20.0	334.0	330.0	30.0	28.0
18.5	282.0	270.0	27.0	25.0
17.5	252.0	240.0	24.0	21.0
16.5	227.0	225.0	15.0	12.0
16.0	225.0	220.0	13.0	10.0
14.0	140.0	140.0	10.0	9.0
13.0	140.0	130.0	8.0	7.0
11.5	105.0	100.0	7.0	6.0
9.3	46.0	38.0	2.0	2.25
9.0	39.0	36.0	2.0	2.1
6.2	17.0	15.0	0.9	0.36
5.2	9.0	8.0	0.25	0.25
2.5	2.0	3.0	0.025	0.064
2.2	1.5	1.75	0.020	0.048
2.0	1.0	1.25	0.018	0.032
1.8	0.75	0.90	0.013	0.018
1.6	0.60	0.70	0.012	0.014
1.5	0.425	0.52	0.010	0.011
1.4	0.350	0.48	0.008	0.010
1.2	0.125	0.210	0.002	0.003

This increase in density is going on simultaneously with the relative decrease in femoral volume as the growth of the limb advances. In an 18-mm. embryo, the volume of the femur constitutes one-fifth that of the entire limb, whereas its density is 1.025. In a 38-mm. embryo, femoral volume is one-sixth that of the limb and its density is 1.055; whereas, in the 50-mm. embryo, the volume of the femur is one-seventh and the density 1.075. The density of the femur in a 20-cm. embryo is 1.6 and the volume is one-sixteenth that of the limb. (Chart B.)

TABLE 2

EMBRYO LENGTH	LENGTH FEMUR	WIDTH FEMUR	WEIGHT FEMUR	VOLUME FEMUR	DENSITY FEMUR
cm.	mm.	mm.	grams	cc.	
20.0	30.0	3.00	2.9	1.8	1.60
18.5	25.0	2.55	2.1	1.6	1.31
17.5	25.0	2.5	1.9	1.4	1.36
16.5	24.0	2.3	1.2	1.0	1.20
16.0	22.0	2.1	0.8	0.69	1.10
14.0	21.0	2.0	0.7	0.64	1.15
13.0	20.0	1.9	0.69	0.60	1.10
11.5	18.0	1.75	0.60	0.54	1.11
9.3	12.0	1.25	0.34	0.28	1.20
9.0	12.0	1.15	0.29	0.27	1.07
6.2	8.0	1.00	0.063	0.06	1.05
5.2	6.0	0.85	0.037	0.035	1.05
2.5	2.95	0.715	0.010	0.010	1.00
2.2	2.55	0.705	0.009	0.009	1.00
2.0	2.40	0.695	0.008	0.008	1.00
1.8	2.15	0.650	0.006	0.006	1.00
1.6	1.50	0.625			
1.5	1.00	0.608			
1.4	0.95	0.459			
1.2	0.88	0.351			

TABLE 3

EMBRYO LENGTH	FEMUR VOLUME	HIND LIMB VOLUME	RATIO OF FEMORAL VOLUME TO LIMB VOLUME	PER CENT OF FEMORAL VOLUME TO LIMB VOLUME
cm.	cc.	cc.		
20.0	1.8	28.0	1:16.0	6.3
18.5	1.6	25.0	1:16.0	6.3
17.5	1.4	21.0	1:15.0	6.6
16.5	1.0	12.0	1:10.0	10.0
16.0	0.89	10.0	1:11.0	9.9
14.0	0.64	9.0	1:14.0	7.0
13.0	0.60	7.0	1:11.0	9.9
11.5	0.54	6.0	1:11.0	9.9
9.3	0.28	2.25	1: 8	12.5
9.0	0.27	2.1	1: 7.5	13.0
6.2	0.06	0.36	1: 6.0	16.6
5.2	0.035	0.25	1: 7.0	12.8
2.5	0.010	0.064	1: 6.4	15.6
2.2	0.009	0.048	1: 5.3	18.8
2.0	0.008	0.032	1: 4.0	25.0
1.8	0.006	0.030	1: 5.0	33.3

With the progressive increase of femoral density the definitive muscles of the thigh become split from the dorsal and ventral premuscle masses. Those myoblasts favorably located along lines of optimum tension for continued muscle differentiation advance in development; whereas, those not so favorably situated revert to embryonal connective tissue. This split into the separate muscles begins with the first compression line forming the hip-joint. Those mesenchymal cells previously drawn out opposite the location of the future joint by the indefinitely outlined blastemal femur retrogress with the progressive clean-cut development of the joints. This is due to the fact that the lines of optimum tension become more definite with the formation of the joints and with the continued development of the definite contour for the femur.

The premuscle mass for the quadriceps extensor (fig. 3) is seen to present a cleavage line opposite the dorsal aspect of the developing hip-joint composed of retrogressive myoblasts. With the continued morphogenesis of the femur and hip-joint, the dorsal premuscle mass is seen to split into its derivatives. The two muscle derivatives clearly seen in figures 4, 5, and 6 are the vastus intermedius and the rectus femoris. Note that the cleavage separating these muscles in the dorsal premuscle mass begins opposite the hip-joint (fig. 4) and progressively advances toward the patella (figs. 5, 6, 7).

The progressive elaboration of the hip-joint advances *pari passu* with the increasing density and the interstitial growth of the femur, together with the developing definitive thigh muscles. The head of the femur advances farther and farther into the acetabulum formed by the ilium, ischium, and pubis. By reference to figures 3, 4, and 5, it is seen that the growing segment forming the femur possesses a greater longitudinal growing force per square millimeter of cross-section than that of the elements forming the acetabulum. By actual measurement at the 20-mm. stage of the embryo, the length of the longitudinally growing femur is 2.40 mm., whereas the acetabulum possesses a depth of 0.195 mm. This is significant, for the force of longitudinal interstitial growth of the femur per square millimeter of cross-



section is twelve times greater than that of the primordial acetabulum. This fact, together with the muscular restrictions to longitudinal femoral growth, and with the fact that the femur is becoming a more stable bar by the appositional growth of bone at its center of ossification, must be considered in order to understand the continued elaboration of the hip-joint by the femur acting like an electric trip-hammer.

With these dynamic points in view, the omnipresent puzzle to the student of anatomy, as to the processes by which one segment of a movable joint possesses the socket and the other the ball, is solved. Joints, according to this evidence, are not hereditary; they are the mechanical resultants of the opposed centers of accelerated growth, segmentally distributed in the apparently continuous, blastemal skeleton.

### 3. INTERPRETATION

It is evident that the ultimate differentiation of muscles, joints, and skeletal components from the uniform mass of mesodermal cells in the hind limb of 10-mm. embryos is dependent upon one of two factors or a combination of both. These factors are, first, an intrinsic self-differentiation of the cells and an extrinsic mechanical interaction due to differential growth. At the present time, the majority of the students of development consider the genesis of the structures enumerated above as a spontaneously hereditary and self-differentiating process, intrinsic to each cell involved. If such is the case, the solution of the problem of development of the limb goes by default. But the writer takes a decided stand to the contrary.

Thoma ('07) considered the first formed bone as the resultant of mechanical factors, but the evidence presented was deductions primarily based on the stress and strain of the mature femur supporting the body weight in the erect position. Thoma was right in his mechanical idea, but his evidence was not convincing. The femur is not formed in anticipation of the stress and strain to which it will be subjected in the future, but is an immediate mechanical resultant of the force of its own interstitial growth and the immediate resistances encountered to this growth.

These resistances may be enumerated as follows: first, the weight of the hind limb; second, the reacting muscular force of elasticity of traction of the surrounding mesenchyme retarded in growth; third, the active muscular contractility manifested during the rotation of the hind limb, and, fourth, the restriction to longitudinal, interstitial growth of the femur at the proximal acetabular accelerated growth center and at the distal tibial accelerated growth center.

These resistances to femoral growth are active and formative during development. They are just as efficient in causing femoral differentiation as the intrinsic accelerated growth of the femoral center itself. Intrinsic growth of the femur and extrinsic resistance are factors *pari passu* in the genesis, growth, and perfected maturity of the femur. Growth and resistance are inseparable; one is just as important as the other. The formative influence of this resistance has been hitherto entirely overlooked.

In embryos 10 to 14 mm. in length, the skeletal condensation of the central core of mesodermal nuclei is purely a mechanical function of position (fig. 2). The syncytial nuclei, located at the center of the limb, have less volume to expand in and a greater resistance to overcome than those located more peripherad. The less volume and the greater resistance are the two factors determining the compactness of the skeletal nuclei.

Once the limb begins to grow rapidly in length, the apparently continuous, compact skeletal core presents two centers of accelerated growth. These centers, dense with nuclei, are separated by transverse zones lighter in texture. The lighter zones are the indefinite lines of demarcation between the centers of accelerated growth of the primordial and segmental blastemal skeleton.

The ultimate external form and internal structure of the various components of the mature skeleton are dependent upon the following factors: First, the varying continuation of the differential intensity of the force of each accelerated growth center of the segmental blastemal skeleton, and, second, the varying resistances encountered by this force. Starting with these two

factors, the muscles, joints, and various degrees in the condensation of the skeleton are mechanical resultants of the interaction of the forces of differential growth.

The intensity of the force of the accelerated growth segment of the femur, together with the resisting reactions of the elongated mesenchyme and the restrictions offered to femoral longitudinal extension by the acetabular and tibial centers at the ends, interact to cause the following definite results: First, the definitive muscles tend to split opposite the joints from the pre-muscle masses along lines of specific optimum tension. Second, the traction to which the primitive muscles are subjected causes an increase in the volume of this tissue. This is manifested by cytoplasmic differentiation of myofibrils and the mitotic reaction of the myoblasts. Third, the definitive muscles now present a definite point of application in reacting to the traction to which they are subjected by the rapid longitudinal growth of the femur. This tends to outline definitely a more stable base or framework on which the muscles act. Fourth, in addition to the force of elasticity of traction presented by the embryonal muscles, the femoral growth center meets resistance at the proximal and distal ends by the acetabular and tibial growth centers, respectively. This tends more and more to outline, then elaborate, the opposed surfaces entering into the formation of the hip- and knee-joints. Fifth, all the above factors converge to cause the femur to assume a characteristic bend. At the center of this bend the mechanical stimulus is first applied, causing the cells to react by forming, first, cartilage, then bone, in the initial differentiation of a progressively more rigid skeleton. Sixth, the differentiation, first, of cartilage at the center of the femoral beam; next, of degeneration of the cartilage, and, finally, periosteal (former perichondrium) bone formation at this center shifts the growing points of the femoral rod to the extremities.

The differentiation of cartilage and its subsequent degeneration at the center of the femoral beam is a critical stage in joint formation. Once a skeletal segment begins to show alteration of its cellular components at the center, they cease to proliferate in the active elongation of the segment. The growth of the

femur in length is then due to the proliferation of the cells constituting its extremities. This terminal growth of a skeletal segment opposes the active growth of the cells of a contiguous skeletal segment.

The first objective evidence in joint formation is significant. There is seen (fig. 4) a linear flattening of the blastemal cells along the line representing the zone of juncture between opposed zones of accelerated growth of neighboring segments. The contour of this linear condensation of cells, which outlines the subsequent position of the incipient or primordial joint cleft, is dependent upon the intensity of the mutual forces of growth opposed in action. That particular skeletal segment presenting the greater intensity of growth force per square millimeter of cross-section will bore into and possess the convex component, constituting a diarthroidal joint, whereas the component presenting the lesser force of growth per square millimeter of cross-section will possess the concave component of a ball-and-socket joint.

The formation of a joint, therefore, is a resultant along the lines of juncture between the zones of accelerated growth of neighboring segments opposed in action. The segment presenting the greater force of growth possesses the convex element, whereas, the segment presenting the lesser force possesses the concave element entering into a movable joint. If the opposed forces are equally distributed between the surfaces of the two segments, a joint with more or less opposed plane surfaces, as the intervertebral joints, is the result.

According to this evidence, muscles, bones, and joints are not hereditary nor do they spontaneously crystallize out of embryonic tissue by some unknown, non-biological method of self-differentiation, but they are the mechanical resultants of an apparently continuous blastemal skeleton, possessing segmental centers of accelerated, longitudinal growth opposed in action. This segmental skeleton is in an environment with which it is syncytially continuous. The effect of longitudinal skeletal growth is traction of the surrounding mesenchyme along the lines of optimum tension evidenced by muscle origin. The perfected

limb represents a continuance of this interaction between skeletal segments and developing musculature until equilibrium is established. This is reached at maturity when the forces of growth are counterbalanced by the resistances to growth. This active interaction of a dominant growth force and its concomitant resistances, playing a dynamic rôle in histogenesis and morphogenesis, has never been heretofore considered in ontogenetic development of the limb.

#### 4. GENERAL CONCLUSIONS

From the facts presented in this paper, the generalization is clearly apparent that the volume of a skeletal segment decreases relatively to the increase of the intensity of the external pressure or resistance. The less the extrinsic resistance to growth early in development, the greater the relative volume of the growing skeletal segment is found to be.

With the decrease in the relative volume of the skeletal segment as development advances, another fact is self-evident, namely, there is an increase in density. The greater the resistances overcome by the growing skeletal segment, the greater the density becomes; conversely, the less the resistance encountered in growth, the less the density, the more gelatinous the consistency. These facts may be summarized in the following laws:

The law of density of a growing tissue: *The density of a growing tissue is directly proportional to the resistances (pressure) encountered during growth.*

The law of relative volume of growing tissue: *The relative volume of a given quantity of growing tissue is inversely as the resistances (pressure) which it bears.*

From the evidence leading to these laws it is conclusive that the various stages of the developing skeleton are resultants of the mechanical resistances to growth which, interpreted, means that cartilage and bone are not self-differentiated, nor are they spontaneously self-crystallized products, but they are the immediate cellular responses to the varying intensity of the stresses and strains produced by resistances (pressure) counteracting the growth of the blastemal skeleton.

## 5. CONCLUSIONS

1. In an embryo 10 mm. in length the hind limb bud is filled with a uniform mass of unmodified mesenchymal cells.

2. The central condensation of the nuclei forming the blastemal skeleton is due to two factors: first, the less volume centrally in which these cells have to expand; second, the resistance of the surrounding mesenchyma.

3. The blastemal skeleton at first grows relatively more rapid in width than in length in embryos 10 to 14 mm. long.

4. With the formation of the periblastemal membrane the subsequent growth of the femur is relatively and absolutely greater in length than in width in embryos 14 to 25 mm. long. This is due to the shifting of the planes of mitosis on account of the compression of the periblastemal membrane. A central skeletal core of accelerated longitudinal growth is now clearly demarked from the peripheral mesenchyme retarded in longitudinal growth.

5. The interaction of these differential growing zones results in a tension of the peripheral mesenchyme. The nuclei of the latter are drawn out in traction, thus resulting in the first step of myogenesis. The direction of the elongated cytoplasmic myofibrils is arranged, therefore, in the line of longitudinal skeletal growth.

6. The formation of skeletal muscle is a dependent differentiation, relying on the accelerated longitudinal growth of the skeleton for its genesis, growth, and continued differentiation. The tension of differential growth is the efficient interacting stimulus to myogenesis in the limb.

7. The following law of direction of the skeletal muscular fasciculi may be formulated from the evidence presented:

*The elongation of the developing muscular fasciculi of the limb is in the direction of the accelerated longitudinal growth of the related dominant skeletal segment. As regards developing muscles in general, the elongation of muscular fasciculi is in the direction of a dominant force extrinsic to the zone of myogenesis.*

## 6. CONCLUSIONS

1. The apparently continuous blastemal skeleton is in reality segmental and composed of centers of accelerated growth opposed in action.

2. By the continued opposition to growth between the contiguous centers of the segmental blastemal skeleton, mechanical compression occurs, revealing the location of the future joint cavities.

3. The contour of the opposed surfaces constituting a joint is dependent on the intensity of the force of growth per square millimeter of cross-section of growing segments opposed in action, together with the force of muscular pull. That segment will possess the ball of a ball-and-socket joint, which possesses the greater force of interstitial growth longitudinally per square millimeter of cross-section.

4. Joints, therefore, are not the cause of skeletal segmentation; they themselves are the mechanical resultants of compressive and shearing stresses of prior centers of accelerated growth opposing each other in action in the segmental blastemal skeleton.

5. *Law of joint formation: The contour of the opposed surfaces forming a joint is dependent upon the intensity of the force of interstitial growth per square millimeter of cross-section of the skeletal segments forming the joint and upon the force of muscular pull.*

## 7. CONCLUSIONS

1. Skeletal condensation, varying through the different degrees of density, beginning with the blastemal period, progressing through the cartilaginous, and terminating in the osseous period, is a direct resultant of the varying intensity of the resistances (pressure) encountered during the period of growth.

2. The resistances to femoral growth are as follows: 1) Weight of the hind limb; 2) reactive force of elasticity of traction of the forming muscles; 3) active muscular pull; 4) opposition to interstitial femoral growth at the ends by the acetabulum proximally and the tibia distally. The evidence presented in this paper warrants the formulation of the following laws:

3. The law of density of a growing tissue: *The density of a growing tissue is directly proportional to the resistances (pressure) encountered during growth.*

4. The law of relative volume of a growing tissue: *The relative volume of a growing tissue is inversely as the resistances (pressure) which it bears.*

5. Cartilage and bone do not self-differentiate, but they are the cellular reactions to the varying mechanical resistances (pressure) encountered by groups of cells in a field of differential growth.

6. The evidence supports the conclusion that resistance to growth is active and formative during development, and that the processes of histogenesis and morphogenesis of the skeleton and muscles are as much dependent upon the mechanical factors extrinsic to the region of the specific developing structure as upon the intrinsic faculty of the modifying tissue to grow. The modifying growing cells receive and respond to the mechanical stimulus. The stimulus, however, is a function of position.

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## PLATE 1

### EXPLANATION OF FIGURES

- 1 Middorsoventral, longitudinal section of hind limb bud of a pig embryo 10 mm. in length.  $\times 40$ .
- 2 Middorsoventral, longitudinal section of hind limb bud of a pig embryo 12 mm. in length.  $\times 40$ .
- 3 Middorsoventral, longitudinal section of hind limb bud of a pig embryo 14 mm. in length.  $\times 40$ .
- 4 Middorsoventral, longitudinal section of hind limb bud of a pig embryo 16 mm. in length.  $\times 40$ .
- 5 Middorsoventral, longitudinal section of the thigh of a pig embryo 20 mm. in length.  $\times 40$ .
- 6 Middorsoventral, longitudinal section of the thigh of a pig embryo 25 mm. in length.  $\times 40$ .
- 7 Middorsoventral, longitudinal section of the thigh of a pig embryo 29 mm. in length.  $\times 40$ .

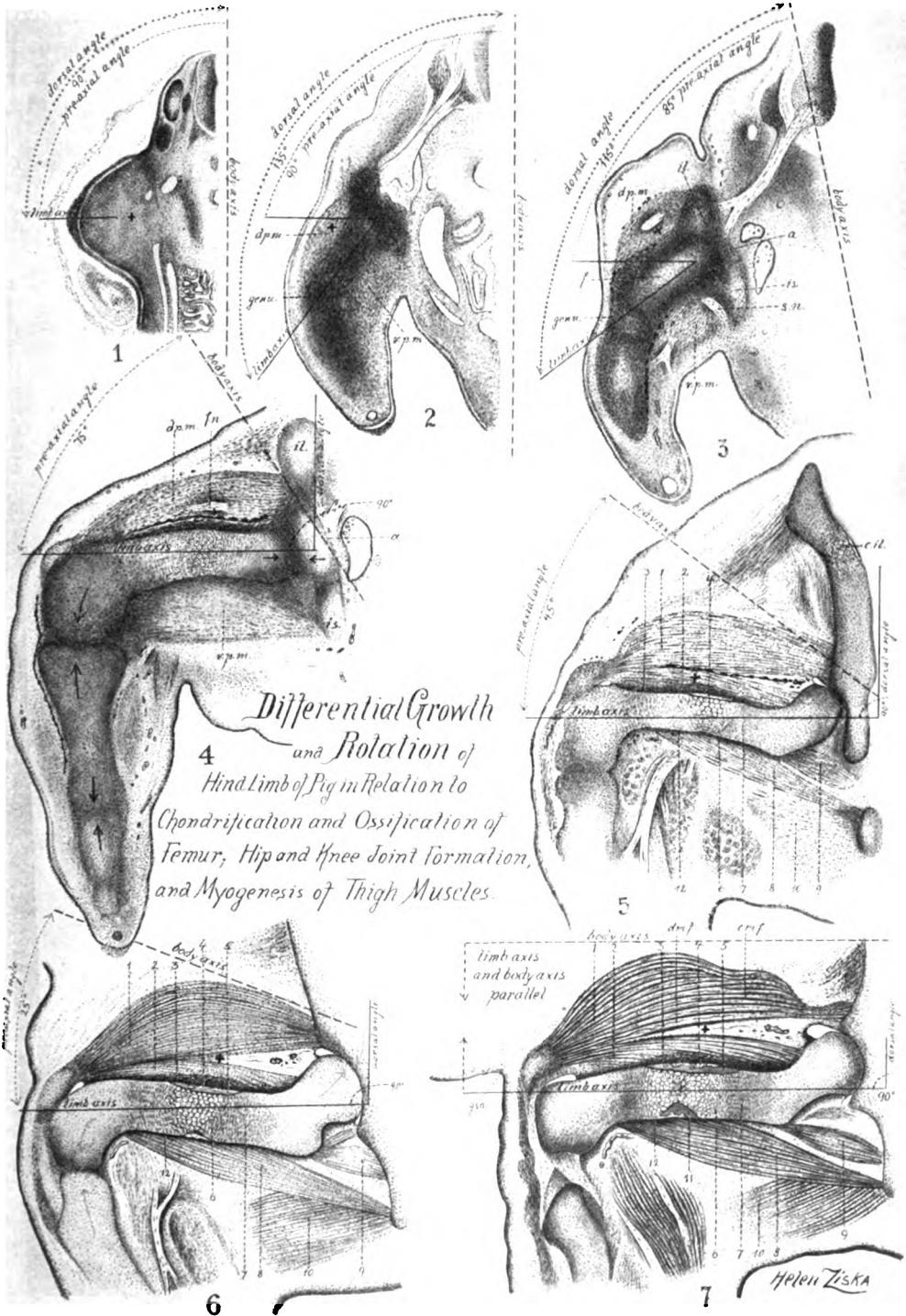
### ABBREVIATIONS

- |   |   |
|---|---|
| <i>d.p.m.</i> , dorsal pre-muscle mass  | 9, pyramidal muscle   |
| <i>v.p.m.</i> , ventral pre-muscle mass   | 10, semimembranous muscle   |
| <i>a</i> , acetabulum   | 11, compressive osseous trabecula   |
| <i>il.</i> , ilium  | 12, compressive perichondrial strain fibrosis (periosteum)  |
| <i>is.</i> , ischium  | <i>dorsal angle</i> , angle formed by a line through the dorsal aspect of the longitudinal axis of the limb with a dorsoventral line through center of hip-joint  |
| <i>s.n.</i> , sciatic nerve   | <i>pre-axial angle</i> , angle formed by pre-axial aspect of limb with lateral body wall. By reference to the various changes in these angles the rotation of the hind limb is exemplified on a plane surface. Since the aspect of the limb cannot change in these figures, the body axis is represented as changing. |
| <i>f.</i> , femur   |   |
| <i>n.</i> , femoral nerve   |   |
| 1, rectus femoris muscle  |   |
| 2, vastus intermedius muscle  |   |
| 3, tensile perichondrial strain fibrosis (periosteum)                               |   |
| 4, Osteogenetic tissue  |   |
| 5, tensile osseous trabecula  |   |
| 6, degenerating cartilage cells arranged along tensile and compressive stress lines |   |
| 7, proliferating cartilage cells in advance of degenerating zone.                   |   |
| 8, abductor magnus muscle   |   |

The attention of the observer is specifically directed to the following facts:

1. The volume of the central condensed blastemal skeleton (fig. 2) occupies relatively more space of the thigh than the femur in figures 4, 5, 6, and 7. It is immediately evident that as development advances the relative volume of the femur to thigh decreases. At the same time, the density increases as exemplified by the progressive deposition of bone (figs. 6 and 7, nos. 5 and 11). The bone on the convex side is drawn out in tension, that on the concave side is compressed. The first deposition of bone, therefore, follows mechanical laws.
2. The width of the femoral segment is relatively greater in figure 2 than that in figures 4, 5, 6, and 7. The femur grows relatively more rapidly in length than in width in embryos 14 to 25 mm. in length. Note that during the accelerated

(Continued on p. 112)

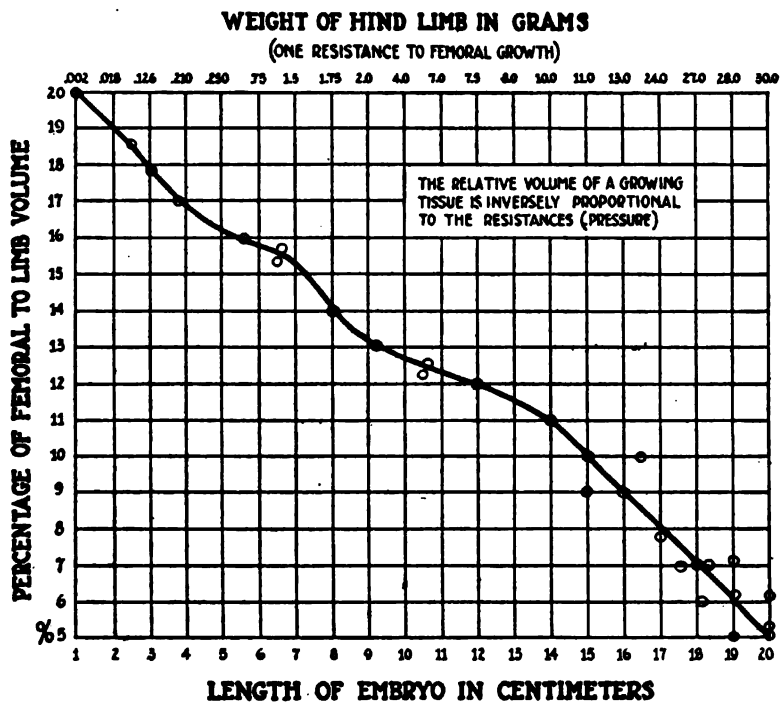


longitudinal growth of the femur the progressive appearance of more and more definite traction lines is seen in the surrounding mesenchyme retarded in longitudinal growth. Along these lines the fasciculi of the developing muscles form, due to the tension of differential longitudinal growth. The determination of accelerated and retarded growth is based on the number of mitotic figures and the compactness of the nuclei per square millimeter of cross-section in a field of differential growth.

3. The force of longitudinal growth of the femur is from ten to fifteen times greater than that of the acetabulum. Compare along the line labeled 'limb axis.' Note, at the same time, the inevitable mechanical effects of muscular pull. These factors, together with the strengthening influence of femoral ossification, determine the location of the convex ball of the hip, ball-and-socket joint, on the femur and not on the region of the acetabulum.

4. The tensile perichondrial strain fibrosis is clearly detected as a limiting membrane, first appearing on the convex aspect of the bent femur (figs. 3, 4, 5, 6, 7, no. 3). Figure 4 represents the limb of a living embryo injected with india ink. Note that the injected capillaries are outside of the perichondrium. This membrane strangles the cartilage cells at the center of the bent femur and later becomes modified into the periosteum from which the osteoblasts proliferate. The osteoblasts, by appositional growth, replace the degenerating cartilage scaffold with a more rigid bony base. These cellular reactions are elicited by the progressive intensity of the strain to which the femur is subjected by the resistances to advancing femoral extension. Longitudinal femoral accelerated growth and extrinsic resistances to this growth are interactions that must be intensively studied in order to appreciate the competition and the resultant products of differential growth.

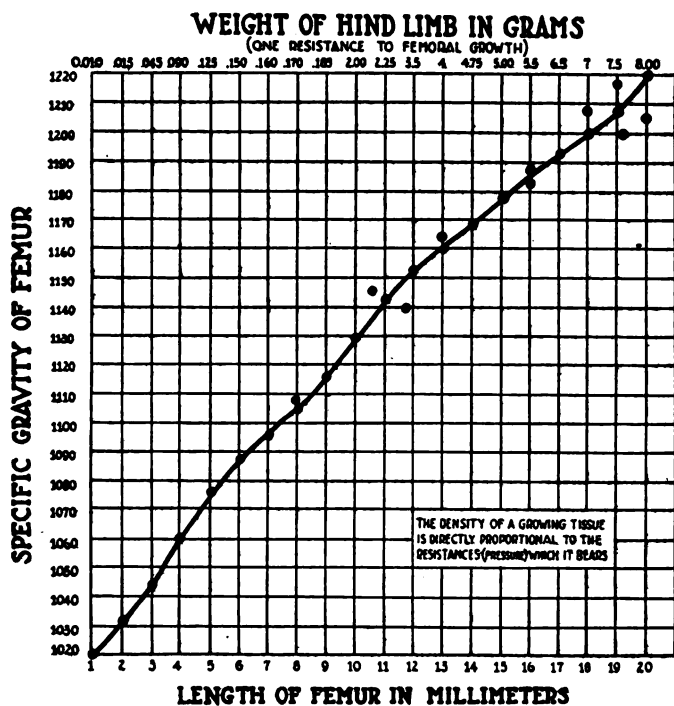
Chart A Curve of relative decrease of femoral volume with increased resistance due to hind-limb weight.



A

Chart B Curve of increase of femoral density with increased resistance due to hind-limb weight. The resistances to femoral growth are as follows: 1) Weight of the hind limb; 2) reactive force of elasticity of traction of the forming muscles; 3) active muscular pull; 4) opposition to interstitial femoral growth at the ends by the acetabulum proximally and the tibia distally.

The specific gravity of these femora was determined by immersion in various concentrations of benzene (sp. gr. 0.879) and chloroform (sp. gr. 1.499). The specific gravity of that solution in which a certain femur would neither rise nor sink was determined by means of the pyknometer; this method gave the specific gravity of the femur. The details of this method will soon be published, with my colleague in biochemistry, Dr. Joseph C. Bock.



B

Resumen por el autor, H. E. Jordan,  
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### Mitocondrias y aparato de Golgi en las células gigantes de la médula ósea roja.

Las células gigantes hemogénicas de la médula roja de los huesos, las llamadas megacariocitos, contienen abundantes mitocondrias granulares y unas pocas de forma bacilar. En los estados jóvenes de estas células predominan las mitocondrias bacilares; en los estados más avanzados existe un exceso de mitocondrias granulares. Las mismas células contienen también un aparato de Golgi relativamente pequeño, el cual aparece bajo la forma de un ovillo más o menos complicado, localizado en la vecindad de los centrosomas. El aparato de Golgi se fragmenta y desaparece en los estados multinucleados avanzados de las células en cuestión. Ambas estructuras pueden observarse en las mismas células después de un tratamiento prolongado con una solución de ácido ósmico al 2%. El aparato de Golgi así puesto de manifiesto es una estructura completamente diferente del retículo canalicular o "trofospongio," descrito primeramente por Retzius en estas células, fijadas con las soluciones de Rabl o Carnoy.

Los resultados de este estudio indican que el llamado trofospongio en estas células, tal como aparece después de la fijación en líquido de Carnoy, es un artefacto; que la red de Golgi consiste de fibrillas varicosas y bastoncitos anastomosados, y que el aparato de Golgi y las mitocondrias son esencialmente prociones, diferentes morfológicamente, de la misma substancia, resultando el primero de la fusión de aquéllas cuando se agregan en la vecindad de la centrosfera.

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## MITOCHONDRIA AND GOLGI APPARATUS OF THE GIANT-CELLS OF RED BONE-MARROW

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TWO PLATES (FOURTEEN FIGURES)

### INTRODUCTION

This investigation was undertaken with the view mainly of adding to our knowledge of the giant-cells of red bone-marrow. Whatever information might accrue with respect to the significance of mitochondria and the Golgi apparatus was a secondary and incidental consideration. Only giant-cells of the hemogenic series come within the scope of this investigation. This work has no reference to the osteoclasts.

In connection with previous studies of the medullary giant-cells, numerous unsuccessful efforts were made, extending over several years, to demonstrate in the extensive cytoplasm of these giant-cells, by various standard technics, mitochondria, an internal reticular apparatus of Golgi, and the trophospongium of Holmgren. A recent more intensive application to this specific aspect of the general problem of the structure and significance of the hemogenic giant-cells, consisting mainly of experiments with modifications of the several fixing fluids in more general use for the demonstration of these structures, has yielded positive results with respect to both mitochondria and the Golgi apparatus, and more or less provisional results regarding the so-called trophospongium.

### MATERIAL AND METHODS

The material employed was the femoral marrow of the rabbit and guinea-pig, both infantile and adult. The method that



finally gave the most satisfactory results was a modification, with respect to time of fixation, of the Kopsch technic. This consists of immersion of very small pieces of the marrow in a 2 per cent solution of osmic acid for a period of four weeks. The tissue must be kept in the dark in an air-tight glass-stoppered vial during the entire period of fixation. This technic reveals both mitochondria and a Golgi apparatus in the same sections and in the same cells.

#### GENERAL

Only two references occur in the literature concerning mitochondria in medullary giant-cells. Dubreuil ('10) records observations on both the osteoclasts (myeloplaxes of Robin) and what he terms the cells of Bizzozero. The latter are identical with the megakaryocytes of Howell. In tissue fixed with Regaud's potassium bichromate-formol solution and stained with iron hematoxylin, Dubreuil describes a great abundance of minute granular mitochondria in the osteoclasts. The same technic revealed occasional, very minute, deeply staining granules in the megakaryocytes. Dubreuil is only willing to say that these latter granules are probably of the nature of mitochondria. Prenant ('11), presumably using the Benda technic, claims to be able, with considerable difficulty, to demonstrate granular mitochondria of varying abundance in what he terms megakaryocytes. He does not state what marrow was used in his investigation. Neither Dubreuil nor Prenant give any illustrations in connection with their descriptions. It is therefore uncertain whether these investigators in all cases of cells called 'cells of Bizzozero' and 'megakaryocytes,' respectively, were actually dealing with the hemogenic giant-cells, or that all the polykaryocytes were actually osteoclasts. One becomes especially suspicious in the case of Dubreuil, for he describes osteoclasts as giant-cells with either a single giant nucleus or with multiple nuclei. The cells of Bizzozero, or megakaryocytes, he correctly describes as cells with a nucleus with many buds, that is, cells with a complex or polymorphous nucleus. As a matter of fact, osteoclasts exist only as cells with more than one, usually many

nuclei, and the hemogenic giant-cells include also cells with many nuclei, that is, polykaryocytes. Prenant apparently considers all multinucleated giant-cells of marrow as osteoclasts, and he describes their mitochondria as consisting of three morphologic varieties: granules, rodlets, and vesicles. These three varieties I can demonstrate also in the hemogenic giant-cells, polymorphonucleated as well as multinucleated.

In two other recent important contributions the results are likewise vitiated by failure to make a distinction between osteolytic and hemogenic giant-cells. Perroncito ('20) speaks of the predominant function of the giant-cells of red bone-marrow as one of phagocytosis, especially for bacteria, erythrocytes, and leucocytes. Pianese ('20), while correctly interpreting the polykaryocyte (of the hemogenic series) as an advanced stage of development of the mononucleated giant-cells ('megakaryocytes'), holds erroneously that it can function also as an osteoclast. Both Perroncito and Pianese hesitate to accept Wright's conclusion that blood platelets are fragments of giant-cell (hemogenic) cytoplasm, but they are unable to furnish any conflicting evidence.

In view of the prevailing confusion with regard to the giant-cells of red bone-marrow in growing and mature bones, it seems desirable to summarize in this connection my general conclusions, based upon an extensive study of osteolytic and hemopoietic foci, regarding two types of giant-cells.

Giant-cells of bone-marrow belong to two ontogenetically, cytologically, and functionally distinct categories: the hemogenic and the osteolytic (Jordan, '18, '21). The former develop, both in red bone-marrow and in the yolk sac, from mononucleated, lymphocyte-like cells, the hemoblasts (Jordan, '18, '19a). They may exist as giant mononucleated cells (giant hemoblasts), and as such are properly called megakaryocytes. They may suffer a nuclear modification, leading to a complex polylobulated 'basket' nucleus, and at such a stage are more correctly designated polymorphokaryocytes. This polymorphous nuclear condition indicates a stage in amitotic nuclear division, and leads to a multinucleated condition, when the cell

may be termed a polykaryocyte (Jordan, '20). The osteolytic giant-cells, on the contrary, arise by the fusion of originally discrete cells of the bone-marrow, including osteoblasts and reticular cells, and are accordingly always and only multinucleated. After Wright's technic, the hemogenic giant-cell, whether mono-, polymorpho-, or multinucleated, and even in the condition of the ancestral hemoblast, is seen to contain a granular endoplasm which takes on a reddish lilac (red to purple) color. The granules are variously designated as metachromatic and azurophil granules. From this granular cytoplasm, by segmentation of pseudopods and by fragmentation of larger areas, are formed the platelets. The cytoplasm of the osteolytic giant-cell does not react in this same way to Wright's technic, nor does it produce platelets (Jordan, '18). Furthermore, the hemogenic giant-cells are not phagocytic. The occasional inclusion of erythrocytes must be interpreted as the result of an intracellular differentiation in these potential erythroblasts, comparable to blood islands, and the included granulocytes as the result of an invasion of the disintegrating giant-cell cytoplasm (Jordan, '20). The chief function of the hemogenic giant-cells is the production of platelets. The osteolytic giant-cells, on the contrary, are distinctly phagocytic. They ingest bone, senile erythrocytes, effete osteoblasts, bone cells, and various other types of débris.

#### MITOCHONDRIA

The giant-cells here to be considered are accordingly only of the hemogenic type, that is, the platelet-forming variety.

An effort was originally made to stain the mitochondria of the giant-cells in fresh preparations of marrow with janus green. Various dilutions were employed, including the standard one of 1 part of the stain to 10,000 parts of a normal salt solution. In such preparations the endoplasm appears granular, the minute granules staining a pale green color. The green-staining granules are presumably mitochondria, but originally such interpretation seemed not quite certain for three chief reasons: 1) the

granules did not stain as intensely as did the mitochondria of certain other tissues similarly treated, e.g., those of the cells of the onion membrane; 2) the granules seemed too minute for mitochondria of such presumably highly differentiated cells, and the possibility presented itself that they might be the metachromatic granules revealed with Wright's technic; 3) it seemed probable that these cells should contain some bacillary and filar mitochondria.

Accordingly, experiments with various fixing and staining technics were next undertaken. The only technic which gave acceptable results was fixation in a 2 per cent osmic-acid solution applied for four weeks. This method reveals abundant granular and annular (vesicular) mitochondria in the cytoplasm of the giant-cells (figs. 5 to 11). An occasional small bacillary mitochondrion also appears, but no filar mitochondria are present in my preparations. It seems, therefore, that the granules revealed by the janus-green solution in the fresh material are actually the mitochondria. The slightly larger size of the mitochondria in the fixed preparations may be the result of a slight swelling action of the osmic acid. Moreover, the metachromatic granules revealed by Wright's technic are more minute, irregular, frequently clumped, and to some extent of a dust-like nature. The relation of the mitochondria to the metachromatic granules has not been determined.

Cowdry's ('14) illustrations of living blood platelets stained with janus green, showing granular mitochondria, support my conclusion that the granules of the giant-cells revealed by the Kopsch technic are mitochondria. The platelets illustrated by Cowdry showing bacillary mitochondria may be of the sort derived from hemoblasts, or even from lymphocytes. In the red bone-marrow of the frog it has been shown that all varieties of leucocytes may produce platelet-like bodies, and it seems altogether probable that in mammals also lymphocytes may to a variable extent contribute to the sum of blood platelets formed predominantly by hemogenic giant-cells ('19 b).

In my best set of preparations mitochondria are revealed only in the hemoblasts and the hemogenic giant-cells. No

mitochondria are discernible in the granulocytes. Nor are the several varieties of specific granules preserved. These granules of granulocytes are represented, with few exceptions, only by vacuoles, the negatives of the dissolved granules. In certain other sets, prepared by the same technic, the hemoblasts and giant-cells, with rare exceptions, lack mitochondria; but in these cases the granules of the granulocytes are deeply stained. They are spherical and annular in shape (fig. 13), and appear identical with the mitochondria of the giant-cells of the other sets of preparations. They can, however, be definitely identified as non-mitochondrial granules by other than morphologic and tinctorial criteria, and not the slightest confusion exists between the cytoplasmic granules of blood granulocytes and the mitochondria of hemoblasts and giant-cells. In certain sets only the former are preserved, in others only the latter. The point of special importance here concerns only the fact that even the Kopsch technic is not specific for mitochondria. This technic stains in the same way also the specific granules of the amphophil (special) and eosinophilic granulocytes, and in such manner as to closely simulate mitochondria.

The large eosinophilic granules stain generally only a deep brown (fig. 13 b), while the granules of the special leucocytes stain black (fig. 13 a). Error and confusion are obviated by the fact that only one of the elements, that is either the specific granules or the mitochondria, stain, with an occasional exception, in the same preparation. This fact emphasizes also the extreme capriciousness of the action of this most favorable mitochondrial technic. Moreover, the reaction to osmic acid suggests that the amphophil and eosinophil granules also contain a lipid constituent. The ring-shaped character of especially the eosinophilic granules results most probably from the solution of the central, less resistant, portion of the granules by this technic, or it may perhaps result from the rarefaction of the center of the granule due to a swelling action of the osmic acid.

In hemoblasts appear only relatively few mitochondria, and a large number of these are of the short bacillary variety (figs. 1 to 4). In general, as the giant-cell progresses in development,

that is, as it passes to the more complex polymorphonucleated, and ultimately to the polynucleated condition, the bacillary and granular types of mitochondria relatively decrease numerically and the vesicular or annular type increases (compare figs. 5 and 7).

Since the Kopsch technic preserves the nuclei only poorly, a minute seriation of successive stages cannot be determined. Accordingly, giant-cells, apparently closely similar with respect to nuclear condition, contain in one case predominantly granular mitochondria, in other cases predominantly vesicular forms (figs. 6 to 8). According to the Lewises, in cultures of young connective tissue ring-shaped mitochondria represent a stage in degeneration and are characteristic of the older cells (p. 395). This agrees with my original conclusion with regard to these giant-cells. Duesberg ('20), however, interprets mitochondria of vesicular form (in older spermatids of opossum) as the result of 'defective fixation.' In living cells in teased preparations he could see no vesicles, but only solid granules. He states further that during this particular period of spermatogenesis the mitochondria are especially sensitive to the action of fixing reagents, while at the same time exhibiting an increased resistance to the dissolving action of acetic acid (p. 64). N. H. Cowdry ('20) finds that in plant cells the form of the mitochondria varies in the same type of cell without discernible cause, developmental, preservative or pathologic. He says that 'it is impossible to find two rootlets, although growing under precisely similar conditions, in which there is not some difference in the mitochondrial contents of cells from similar portions.' He finds 'also that there is much variation in the manner in which mitochondria react to experimental conditions in different parts of the same rootlet and also in rootlets of different stages of growth' (p. 197). Nicholson ('16), on the contrary, records for certain types of neurons of the brain of white mice a fairly specific qualitative difference in the mitochondrial content, as well as a fairly constant variation in form of mitochondria in different parts of the same cell (p. 342). Moreover, in the eosinophilic leucocytes preserved in the marrow fixed with osmic acid, many

of the granules appear vesicular (fig. 13). I come to the tentative conclusion that as these giant-cells progress in development beyond the hemoblast stages bacillary mitochondria give way to granular, and that in the older stages of the polymorphokaryocytes the granular mitochondria react to this technic in such a manner as to give the appearance of a vesicle or ring. Apparently the less resistant center of the eosinophilic granule, and of the granular mitochondrion, is dissolved or rarefied by the osmic acid of this technic.

The mitochondria obviously increase enormously in number as the young giant-cells grow in size, but I have seen no indication of the manner of mitochondrial formation. The most obvious conclusion would seem to be that in these cells new mitochondria arise from out of the cytoplasm rather than by segmentation of preexisting mitochondria. Only occasionally are the mitochondria arranged in definite lines, simulating in this condition varicose or segmenting fibrils (fig. 9).

In certain giant-cells the segregation of the mitochondria within the endoplasm is striking (figs. 5 and 7). In this respect the mitochondria are disposed like the metachromatic granules shown in preparations according to Wright's technic. In these cases there appears a clear, narrow, non-granular exoplasmic layer. However, no evidence appears in my preparations bearing on the question of the genetic relation between mitochondria and the metachromatic granules. The number of mitochondria also varies greatly in different cells (compare fig. 7 with figs. 10 and 11).

As regards mitochondria, therefore, the definite result accrues from this study that the hemogenic giant-cells of red bone-marrow are characterized by a type of mitochondria predominantly of the small granular form.

#### THE GOLGI APPARATUS

The sole recorded observation on the internal reticular apparatus of Golgi in giant-cells of red bone-marrow is a brief reference by Retzius ('01). Retzius used the bone-marrow of young

rabbits and cats. At least in so far as he dealt with the bone-marrow of rabbits, he was almost certainly concerned exclusively with the hemogenic giant-cells. His results may therefore be properly compared with mine for this tissue.

Retzius used as fixing fluids Rabl's mixture (sublimite-picric-acetic-acid) and Carnoy's fluid. In preparations stained with iron hematoxylin and various counterstains, including eosin, he claims to be able to demonstrate the Golgi apparatus.

Since Retzius' contribution includes no illustrations, and since the modified Kopsch technic gives a picture very different from that described by Retzius, I repeated Retzius' technic with a view to observing the specific appearance that he interpreted as a Golgi apparatus.

The condition that seems least likely to represent a fixation artifact, while at the same time corresponding fairly closely to the description of Retzius, appears in the preparations fixed with the Carnoy fluid no. 1 (absolute alcohol 60 cc., chloroform 30 cc., glacial acetic acid 10 cc.). Rabl's mixture, as also the Carnoy's fluid no. 2, produces results which are obviously exaggerations of the condition seen in the tissue fixed with the unmodified Carnoy's fluid, and which seem clearly to be fixation alterations. As illustrated in figure 12, the Carnoy fluid (no. 1) discloses a collection of anastomosing canals, in general parallel with the surface, and apparently opening onto the periphery. The system, as revealed by changing the level of focus, is rather more complex than could be shown to advantage in the illustration. This system answers to Retzius' description of a structure of 'peculiar canal-like character apparently similar to the trophospongium described by Emil Holmgren in spinal ganglion cells.' Influenced, however, by Heidenhain's idea regarding the function of these giant-cells, namely, 'that they serve solely to imbibe and alter proteids taken either from the lymph or the blood stream to be again returned to them' (cited from Retzius), he inclined to interpret this system of canals as 'similar to the secretory canals of gland cells rather than nutritive canals.' He states further that 'these canals of giant-cells appear to be a mechanism for the elimination of products of metabolism of these



cells.' This conclusion he regards as favored also by the fact that the canaliculi open upon the periphery.

It seems important to record the fact that the appearance of the cytoplasm differs greatly in different cells of my preparations fixed with Carnoy's fluid. In some cells it appears homogeneous or very finely granular, without indication of either canalicular network or mitochondria; in other cells the cytoplasm is filled with black-staining granules and varicose threads, the granules being apparently identical with the mitochondria of the Kopsch preparations. In the cells in which the cytoplasm is canalized as above described, the black-staining granules and threads are either extremely rare or entirely lacking. I am unable to decide, on the basis of available data, whether this difference in the constitution of the cytoplasm represents a genuine functional and structural difference or a variable effect of the fixing process, or whether it is correlated with a difference in the stage of development, showing itself in a difference of reaction to the Carnoy mixture. The latter interpretation seems to me the most probable.

It is of prime interest that Retzius states that he was unable to demonstrate 'this canalicular apparatus of giant-cells by the application of Golgi's chrom-osmium-silver method so favorable for revealing this apparatus in gland cells.' This was also my experience. In fact, I was unable to demonstrate such a canalicular apparatus of Holmgren or a genuine Golgi reticulum by any of the standard methods, other than the Kopsch method in the case of the Golgi net, in general use for the preservation of these cell contents.

In my experiments with various fluids for the demonstration of mitochondria and the Golgi apparatus in the giant-cells of bone-marrow I used in practically every attempt also testicular tissue. With the Kopsch method it was a simple matter to demonstrate in the same sections of the testis (rabbit and opossum) structures identical with what have been described and illustrated many times by many workers with various technics, both mitochondria and Golgi apparatus (fig. 14). With the time modification the Kopsch method revealed similar structures

in the giant-cells of the red bone-marrow (figs. 5 and 7). I therefore feel justified in concluding that a structure, entirely different from that described by Retzius, and agreeing in essential particulars with the reticular apparatus of Golgi described in many other cells, occurs typically in these giant-cells of red marrow. In short, the technic employed by Retzius does not reveal the Golgi apparatus, but does disclose a system of anastomosing canals that may correspond to what Holmgren has described as a trophospongium; but which seem to me, at least in this tissue, to be of the nature of a fixation artifact.

The conception that I have acquired of this 'canalicular apparatus' in these giant-cells of my preparations fixed in Carnoy's fluid is that of a result produced by a condensation (coagulation) of the cytoplasm along certain planes, leaving thus an irregular system of intervening layers of more fluid substances simulating canals. In this field of cytology it is especially unsafe to make unqualified statements, and I would not be understood to even intimate that all so-called trophospongium and canalicular apparatus is artificial, either the result of fixation or temperature changes. But I feel quite in accord with Duesberg's ('20) conclusion that the closed, localized Golgi apparatus is a structure distinct from the diffuse canalicular apparatus, opening at certain points on the periphery, commonly described as Holmgren's trophospongium, in spite of Holmgren's ('14) insistence that these two structures are one and the same thing. At any rate, in these giant-cells the canalicular apparatus revealed by the Carnoy's fluid, and first described by Retzius, has no direct relation to the much smaller and sharply localized network as revealed by the Kopsch technic. Moreover, this network, which I am designating as the Golgi apparatus, consists, as revealed after the osmic-acid treatment, of solid elements, not of canals.

The Golgi apparatus of the hemogenic series of giant-cells appears already in the hemoblasts as a closed loop of somewhat irregular form, closely applied to the surface of the centrosphere (figs. 1 to 4). I have not succeeded, as I had hoped to do, in tracing the detailed steps of its development concomitant with the assumption of the polymorphonucleated and the multi-

nucleated conditions of these giant-cells. But the Golgi apparatus clearly enlarges and becomes more of the nature of a closed network, always located close to the area which contains, as deduced from other preparations, the fragmenting centrosomes (fig. 5). In the later polynucleated stages, determined as accurately as the poorly preserved nuclei of this technic will permit, the reticular apparatus is lacking. It apparently breaks up and disappears coincidentally with the partition and disappearance of the centrosomes (fig. 9).

The question of relationship between Golgi apparatus and mitochondria, seeing that they react in similar fashion to the osmic acid, presents itself for consideration. On the basis of staining reaction in osmic acid, one might conclude that Golgi apparatus and mitochondria are only different portions or different morphologic expressions of a chemically identical, certainly similar, cytoplasmic element. Such conclusion would seem to receive support from the fact that, when closely analyzed, the Golgi apparatus, especially in male germ cells, appears to be composed of elementary rods and granules, fused into a more or less intricate and continuous network; and further, from the additional fact that the mitochondria tend to aggregate about the centrosphere, the vicinity of which is frequently the site also of the Golgi apparatus (fig. 14).

In the hemoblasts and their giant-cell derivatives the Golgi apparatus is located close to the position of the centrosome (figs. 1 to 4). If the mitochondria which have congregated about the centrosphere fused, a network would presumably result very similar to the network of this location called the Golgi apparatus (fig. 5). However, there is no crucial evidence to prove that the granules scattered throughout the cytoplasm, and which appear identical with mitochondria, are not actually derived by fragmentation from the Golgi apparatus. Determination of relation, on the basis of staining reaction to osmic acid, is made to appear still more uncertain when we recall the close similarity, amounting apparently to an identity, between the special (amphophil) and eosinophilic granules of the granulocytes of this marrow and the mitochondria of the giant-cells.

Both the conclusion of an identity of these elements and that of a dissimilarity have had numerous supporters. Perroncito ('10), Cajal ('14), and Gatenby ('19), for example, regard these elements as genetically distinct structures; Deineke ('14) and Monti ('15), among others, regard them as only different morphologic expressions of the same substance. Duesberg ('20) originally regarded the two elements as different structures. In his latest consideration of this question, he seems unwilling to state a definite conclusion, while apparently inclining to a belief in a close genetic relationship.

When one recalls the illustrations (figs. 5 and 9) of the Lewises ('15) showing the granular mitochondria of connective-tissue cells in tissue cultures fusing to form bacillary and filar mitochondria, which latter anastomose to form a complicated network, one sees a series of events which has been actually observed in living cells paralleling in a measure the steps that would most conceivably be taken by mitochondria in the process of transformation into a Golgi apparatus.

We know as yet too little definitely about the chemistry of the Golgi apparatus, and of the chemically different substances and mixtures which may give an identical color reaction with osmic acid, to warrant anything approaching a final statement with regard to the genetic relationship between the mitochondria and the Golgi apparatus. In this material, considering the practically innumerable granules in the cytoplasm, and the localized distribution and the relatively small size of the Golgi apparatus, we are on safe ground when we interpret the granules of the giant-cell cytoplasm as mitochondria, rather than as portion of a diffuse Golgi apparatus.

As regards the physical constitution of the Golgi apparatus, Pappenheimer ('16) inclines to accept the conclusion of Cajal that the apparatus consists of a system of canaliculi which are filled with a lipid-containing substance. The canals are thought to be relatively rigid and of permanent form in certain fixed cells, but more plastic in secretory and embryonic cells (p. 138). Duesberg ('20) expresses some skepticism regarding such an interpretation (p. 76). Bensley ('14) interprets the apparatus

in the islet cells of the pancreas of the guinea-pig as a network of canals (p. 365), while Gatenby ('19) considers both Golgi apparatus and mitochondria to consist of a substance of living protoplasm denser than the surrounding medium in which they lie (p. 115).

E. V. Cowdry ('21) suggests the terminology 'reticular material' for the so-called Golgi apparatus, in view of our limited knowledge regarding its intimate structure. He describes it as a restricted area of fluidity in the cytoplasm (p. 8). His observations on this 'material' in the erythroblasts, leucocytes and lymphocytes of the red marrow of the guinea-pig lead him to the tentative conclusion that it is of a 'watery consistency, being even more fluid than the general ground substance, and is probably changing its form continually as it plays its obscure part in the activities of the cell, for no two networks as seen in fixed preparations of blood cells, or any other kind of cell, are exactly alike' (p. 7).

My own observations incline me to an interpretation of the Golgi apparatus in terms of solid fibrils rather than of canaliculi; at least 'solid' in a manner similar to the constitution of the mitochondria and that of the eosinophilic granules of the leucocytes. In some of the preparations the latter granules are deeply stained by the osmic acid, like the mitochondria of other preparations, and appear solid. In other preparations, where the mitochondria have become visible by the reaction to osmic acid, the eosinophilic granules have been dissolved, leaving only spherical vacuoles. In a similar fashion, the presumably solid Golgi net may be conceived to become in certain instances a system of canaliculi by the solution of fibrils, leaving behind the cytoplasmic mold of which the 'fibrils' formed the cast. The changing form and character of the Golgi apparatus would be the consequence of the continual addition of new portions (mitochondria?) and the continual destruction or resorption of other portions.

## SUMMARY

1. The hemogenic giant-cells of the red bone-marrow, the so-called megakaryocytes, contain abundant granular and a few bacillary mitochondria. In the earlier stages the bacillary forms predominate, in the later stages the granular are in excess.

2. The same cells contain also a relatively small Golgi apparatus in the form of a more or less complicated skein localized in the vicinity of the centrosomes. The Golgi apparatus fragments and disappears in the later multinucleated stages of these cells.

3. Both of these structures can be demonstrated in the same cells after prolonged treatment with a 2 per cent solution of osmic acid.

4. The Golgi apparatus thus demonstrated is an entirely different structure from the canalicular network or trophospongium first described by Retzius in these cells fixed with either Rabl's or Carnoy's solutions.

5. The results of this cytologic investigation of these giant-cells in the red bone-marrow of the rabbit and the guinea-pig lead to the tentative conclusions that the so-called trophospongium of these cells as demonstrable in Carnoy-fixed tissue is an artifact, that the Golgi network consists of anastomosing varicose fibrils and rods, and that the Golgi apparatus and mitochondria are only morphologically different portions of the same substance, the former resulting from a fusion of the latter while aggregated in the neighborhood of the centrosphere.

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## PLATE 1

### EXPLANATION OF FIGURES

All figures were drawn with a Leitz 1/16 objective, a no. 1 ocular, and a camera lucida. The magnification is about 1600 diameters.

1 Hemoblast. The nucleus appears as a central, pale, homogeneous, circular area. Above the nucleus is a small, clear, circular area, the centrosphere, closely applied to which at the left is a deeply staining, collapsed loop-like structure, the Golgi apparatus. Throughout the cytoplasm, for the most part near the nuclear wall, are scattered granular and bacillary mitochondria.

2 Hemoblast. In this cell the Golgi apparatus encircles the centrosphere, and appears to consist of several fused rods and granules. The cytoplasm contains a few vesicular mitochondria.

3 Later hemoblast, or young mononucleated giant-cell, apparently in ameoboid motion. The mitochondria are largely of bacillary form. Along the upper border the mitochondria are aggregated into spherical group suggesting either a forming or a disintegrating Golgi apparatus.

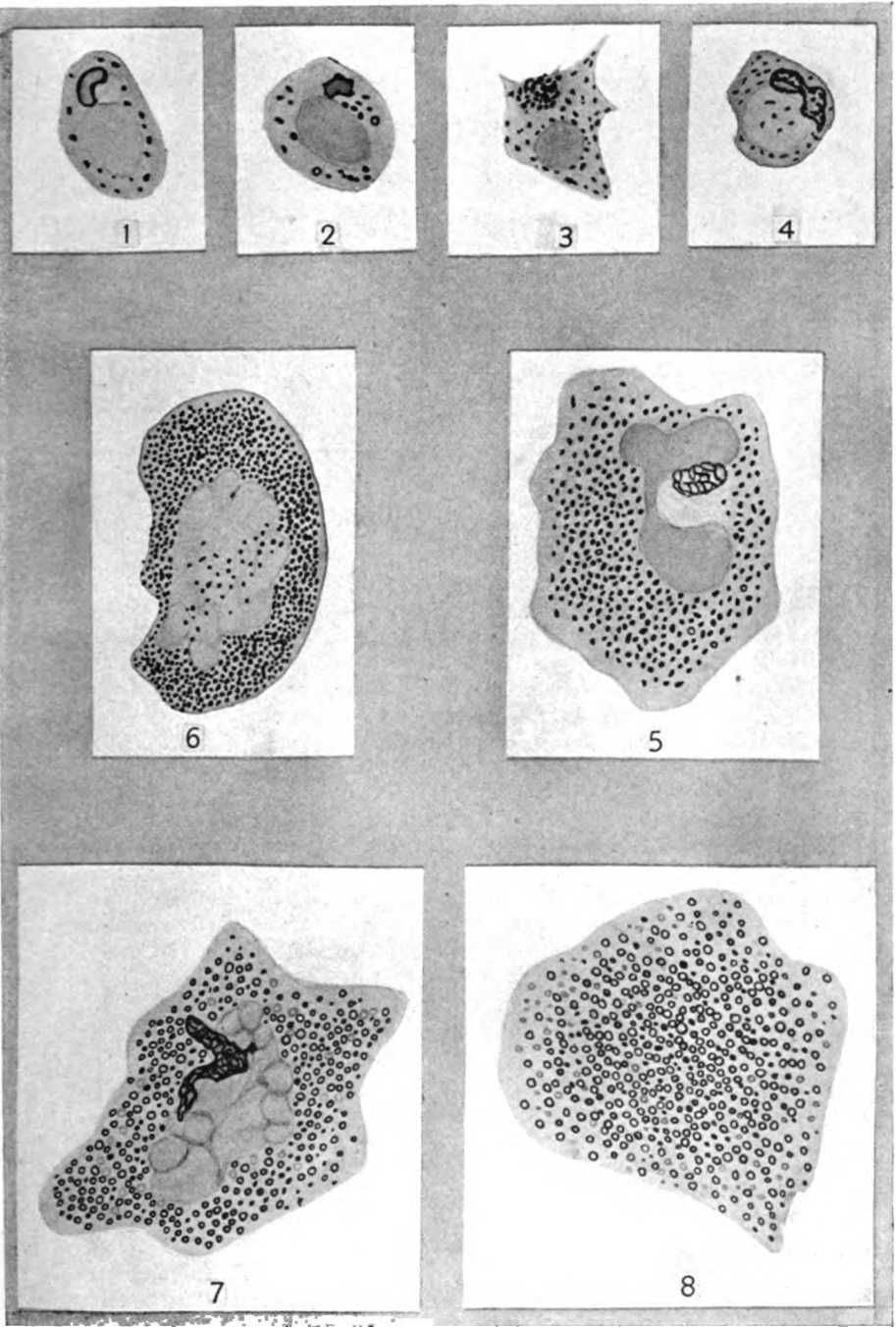
4 Hemoblast. The Golgi apparatus consists of a large, apparently continuous, bilobed net, closely applied to the nucleus. The mitochondria are predominantly of the bacillary form. A few mitochondria are shown scattered over the central nucleus.

5 Young polymorphokaryocyte. The enlarged nucleus is still relatively simple, consisting of two large lobes connected by a narrow bridge of nuclear material. Within the nuclear concavity, slightly to one side, the usual location of the now fragmenting centrosome, is situated an intricate network of delicate and deeply staining threads, the Golgi apparatus. The cytoplasm contains mostly short, rod-shaped mitochondria, with a few granules and vesicles. A narrow, clear, non-granular exoplasmic layer is here very conspicuous.

6 Older polymorphokaryocyte (megakaryocyte). The Golgi apparatus is not here shown. The cytoplasm is packed with exclusively granular mitochondria.

7 Older hemogenic giant-cell with complex polymorphous nucleus (polymorphokaryocyte). The Golgi apparatus is shown to the left. It consists of an intricate skein of deeply staining threads. The mitochondria are predominantly vesicular.

8 Section of preceding cell, 15 $\mu$  beyond the section of figure 7. The mitochondria are very abundant, including both granules and vesicles.



## PLATE 2

### EXPLANATION OF FIGURES

9 Later stage of hemogenic giant-cell. At opposite poles of the nucleus are located two small, irregular, deeply staining networks, presumably the remnants of the originally single Golgi apparatus. Each moiety of the Golgi net lies peripheral to a cytoplasmic vacuole. The vacuole may have been produced by a contraction of the Golgi nets during dissolution or during fixation. The mitochondria are exclusively of the minute granular type. Above, in a clearer circular area, the mitochondria are so aligned as to simulate varicose and segmenting threads.

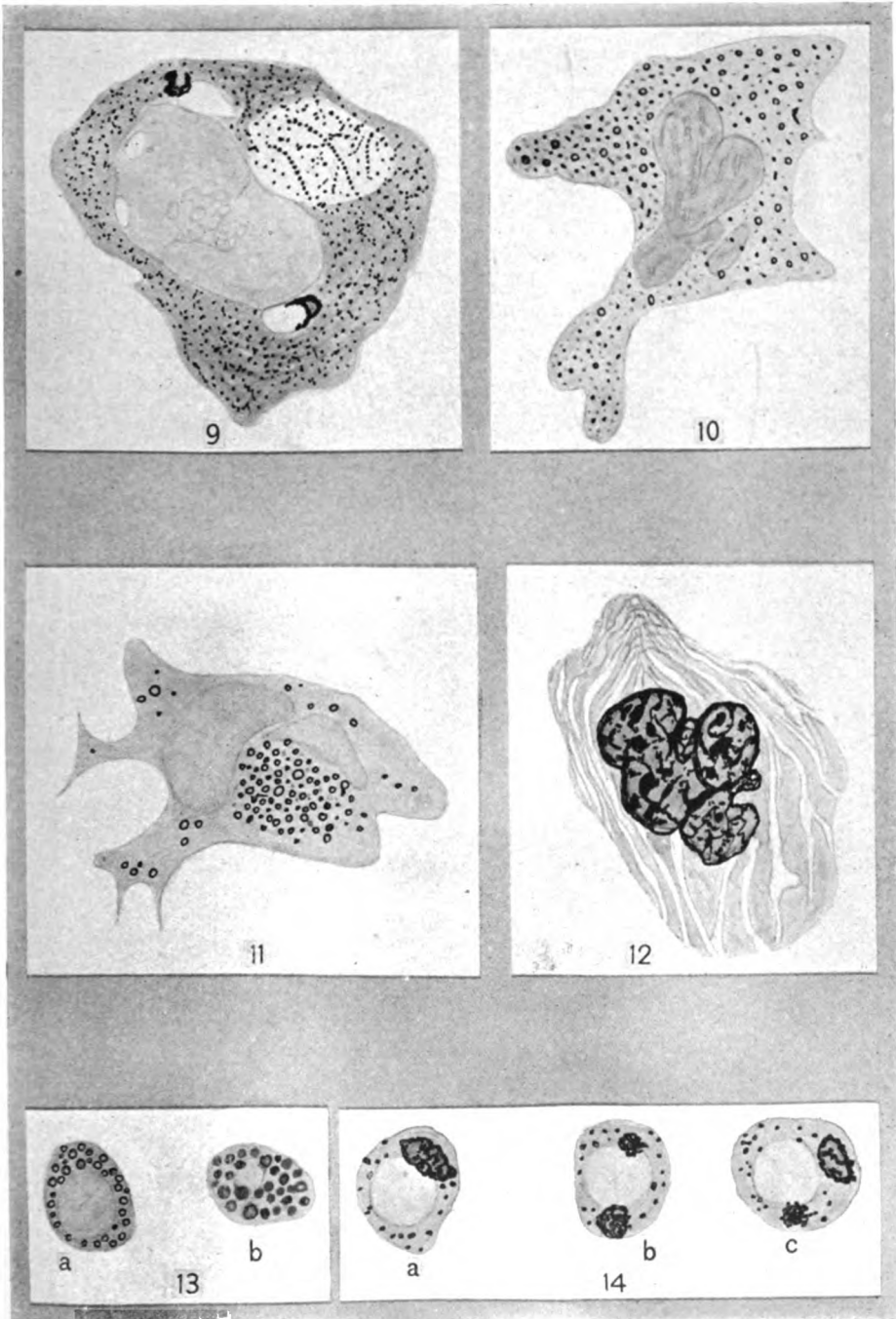
10 Polykaryocyte of the hemogenic series. Several blunt pseudopods occur on this cell. The mitochondria include short rods, granules, and vesicles.

11 Similar giant-cell with relatively few mitochondria, the majority in the form of vesicles.

12 Giant-cell from marrow of rabbit, fixed with Carnoy's fluid no. 1 and stained with iron hematoxylin. The cytoplasm contains an anastomosing system of canals, corresponding to the so-called trophospongium of Holmgren. Similar appearances in these cells fixed in the same way were described by Retzius as a Golgi apparatus of the cells. The presumption would seem to be justified that in these cells the 'canals' are fixation artifacts.

13 a and b Special (amphophil) and eosinophilic granulocyte, respectively, of marrow of the guinea-pig as they appear in Kopsch preparations. Note the similarity of the granules to mitochondria.

14 a, b and c Primary spermatocytes of testis of rabbit, preserved by the Kopsch method. The central nucleus appears as a pale, circular area. The cytoplasm contains a number of granular and bacillary mitochondria. About the centrosphere is located a deeply staining network of varicose threads, presumably formed by fusion of rods and granules. In b and c occurs a third structure simulating somewhat a smaller Golgi apparatus. It is usually located at the nuclear pole opposite to that occupied by the centrosphere and the Golgi apparatus. It may however be located close to the larger network (as in c), and some cells contain two such groups. This third structure is tentatively interpreted here as resulting from the aggregation of mitochondria in certain regions.





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Resumen por el autor, T. H. Bast.

Estudios sobre la estructura y multiplicación de las células óseas  
facilitados por una nueva técnica.

En el estudio de un tejido es de gran importancia que la fijación reproduzca exactamente la estructura viva. En el estudio de las células óseas los mejores resultados pueden obtenerse en preparaciones hechas del siguiente modo: Una lámina delgada o delgadas astillas de hueso se fijan en alcohol, se tiñen en violeta de genciana, deshidratando mediante el alcohol y pasándolas por benzol; y después de separar cuidadosamente el periosteo, se montan in toto en bálsamo neutro. Tales preparaciones presentan la cromatina nuclear intensamente teñida, el citoplasma más claro y una matriz ósea incolora. Este método elimina el empleo del ácido que siempre ejerce una acción disgregante sobre el tejido. Permite el estudio del hueso in toto, facilitando el adquirir un concepto más verdadero de las relaciones y forma de las diversas estructuras, una virtud que no poseen los cortes, a menos que sean seriados. La matriz ósea de estas preparaciones es tan clara y transparente que las granulaciones citoplásmicas, centrosomas, prolongaciones protoplásmicas y sus anastomosis, la ausencia de espacios lacunares y canaliculos, la forma y estructura del núcleo y el tipo de división nuclear, que es siempre la amitosis, pueden determinarse sin dificultad. Esta multiplicación de las células junto con el hecho de que en el hueso muy joven están más próximas entre sí que en el más viejo indican que las células óseas no son corpúsculos inactivos, sino que están relacionados de alguna manera con la producción de tejido óseo.

Translation by José F. Nonides  
Cornell Medical College, New York

# STUDIES ON THE STRUCTURE AND MULTIPLICATION OF BONE CELLS FACILITATED BY A NEW TECHNIQUE

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ONE PLATE (SIX FIGURES)

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## INTRODUCTION

According to the generally accepted view, a bone cell may be defined as a modified, functionless or senile osteoblast. The osteoblast or bone-former is a characteristically changed fibroblast. Certain fibroblasts become rounded and are transformed into osteoblasts which arrange themselves on the surface of the bone spicules where they deposit osseous substances. The origin of the fibroblast is somewhat different in the two types of bone. In membranous bone the fibroblasts arise in situ from the mesenchyme while in cartilaginous bone they are carried by the osteo-



genetic buds into the areolae of Sharpey from the perichondrium. T. W. Todd, however, believes that the fibroblasts of cartilage bone arise in situ. Thus he says: "Osteoblasts do not enter skeletal tissue along the blood vessel's tracks, but are fibroblasts or connective tissue cells which have undergone certain characteristic modifications and may not have passed through the chondroblast stage." This view is also held by Macewen.

The stages passed through by the fibroblast in becoming an osteoblast, do not directly concern us, the transformation of the osteoblast into a bone cell is the point of immediate importance. According to current views the osteoblast deposits bone only on that surface which is in contact with bone spicules. When one of these osteoblasts becomes aged, worn out, or inactive it is gradually surrounded by the osseous product of other osteoblasts which remain active. At this stage the term osteoblast (bone former) no longer applies and the name bone cell is used. Thus bone cells are passive protoplasmic bodies lying in bone pockets called lacunae. It is to this cell lying within the lacunae that we shall turn our attention.

#### TECHNIQUE

The material for this paper presented itself in an entirely unlooked for manner. While investigating the glands of a dog's nose the thin lamina of bone of the turbinates and the ethmoid attracted attention. Curiosity led to a number of experiments which proved interesting. After trying many stains and manipulations in order to obtain differential preparations the following procedure gave satisfactory results:

1. Fix small pieces of thin bone in 95 per cent alcohol.
2. Wash in water.
3. Stain for 8-24 hours in a dilute aqueous solution of Gentian Violet. (The stain should be diluted until it is transparent when viewed in a test tube.)
4. Dehydrate as rapidly as possible in 75 per cent, 95 per cent and absolute alcohol.
5. Clear in benzol.

6. Place in a watch glass of benzol and carefully scrape off all of the periosteum under a binocular microscope.

7. Mount in balsam.

The advantage of Gentian Violet over other stains lies in the fact that bone cells stain very intensely while the matrix remains unstained. The deep stained cells with all their protoplasmic processes within the clear white bone gives a very striking picture. The older the bone the greater the contrast. This is probably due to the fact that the bone cells are farther apart and the abundant and more compact bone is less prone to absorb the stain. Among other stains that were used, neutral red gave similar results but the contrast is not as great because the cells are not stained as intensely as with Gentian Violet.

When bone from which the periosteum has been removed, is stained the picture is not as clear. The denuded bone apparently absorbs some of the dye. With the periosteum intact the stain will not diffuse through the osseous matrix. Before attempting to study such sections it is very important that the periosteum be carefully removed. The outer or limiting membrane is easily removed but the inner part of the periosteum is not membranous but more granular and adheres firmly to the bone. It is this inner layer that takes the stain deeply and unless removed obscures the details of the bone itself.

The bones employed in these investigations were thin lamina from the turbinates and ethmoid bones of both young and adult dogs; the parietal and nasal bones of young cats and rabbits; the parietal bones of mice and rats; thin pieces of young rat femur; and the thin lamina of bone from the human ethmoid and from the walls of the human sphenoidal sinuses. A complete series of parietal bones of 15 rats ranging from one to fifteen days old were studied. This series was taken from two litters, one of eight and the other of seven young. Fresh bone was also studied with good results. The refractive indices are sufficiently different to permit one to distinguish between cells and matrix. All of these preparations even the parietal bones of half grown rats and three week old kittens are thin and transparent enough to permit examination with oil immersion lenses.

The value of this type of preparations may be summed up as follows:

1. Bones need not be decalcified to obtain preparations fit for oil immersion study.
2. No sections need be cut but bone can be studied in its entirety.
3. Bone can be examined fresh with water immersion lenses.
4. The bone cell and its processes can be viewed with great clearness without being subjected to the severe and distorting action of acids.

#### OBSERVATIONS ON BONE CELL STRUCTURE

We shall not describe the osteoblast but the bone cell only. However, it is often rather difficult to determine where the osteoblast stage ends and the bone cell stage begins. Lewis and Stöhr describe the beginning of the bone cell as follows; "Osteoblasts . . . . form bone only along that surface which is applied to the matrix. As the strand of bone grows broader through their activity, it encloses here and there an osteoblast, which thus becomes a bone cell." Since this seems to be the generally accepted view we shall take as a criterion the point where the rounded osteoblast is replaced by the star-shaped body completely surrounded by an osseous matrix.

The pieces of bone for this study varied in thickness from one to four times the thickness of the bone cells. In such preparations cells are found at various levels. With a 16 mm. objective the cells appear very small, and since a low power lens permits a considerable depth of focus several layers of cells are brought into view often confusing the true relationship. The 4 mm. and the oil immersion lenses are used to better advantage, the oil immersion giving the most satisfactory results. The depth of focus is much less than the diameter of the cell and so a clear focus can be obtained on any part of the cell. The structures lying below or above a given focus appear quite transparent when a strong light and an open diaphragm are used. Only those cells which lie in the same plane of focus are visible.

By slowly changing the focus the relation to other cells at other levels can be established. Since the osseous matrix is unstained or only slightly tinted, the deeply stained bone cells with their anastomosing protoplasmic processes produce the picture of a syncytium.

*a. Shape and size of bone cells*

The shape of the bone cell is usually stellate with a more or less elongated body, but difference in shape depend on the age of the cells. Cross sections of the parietal bone show that the bodies of bone cells are not flat but round or oval. Very young cells are more rounded much like the osteoblast and the processes are short or sometimes even absent. In older cells the stellate shape is very marked. The cell body varies from 10 to 18 micra in diameter although some polynucleated cells measure more. In young cells the average diameter is greater than in older cells. The young cells are usually preparing to divide, and this may be the reason for the difference. This seems to be substantiated by the fact that in very young parietal bone of the rat some groups of cells are very large while other groups are rather small. The nuclei of the large cells usually have divided or are about to divide (fig. 6). The groups of smaller cells show signs of having passed through a stage of rapid division (fig. 2). Both of these groups of young cells can be found near the periphery of the growing parietal bone. The difference in size, however, is not entirely explained by the stage of proliferation because a difference in size also exists in older cells of different ages. In the one day old rat the cells at the center of the parietal bone—these are the oldest cells—are large, measuring from 15 to 16 micra in diameter (fig. 4). On the other hand the cells near the center of a 15 day old rat measure about 12 to 13 micra (fig. 3). Figures 3 and 4 also show the interesting fact that the oldest cells of the 15 day old rat are about 20 micra apart while those of the one day old are separated by only 16 micra. The difference is usually greater than is shown by these figures. This difference can also be seen between the peripheral and central cells of a given young bone. Thus the central

cells of a one day old bone (fig. 3) are 16 micra apart while those more peripherally located are separated by only 4 micra, (figs. 2 and 6). Figures 2, 3, and 6 are drawings from the parietal bone of a rat fifteen days old.

A series of young rats, up to three and one-half weeks old including the series already mentioned, bear out these observations. The younger cells as a rule are large and close together while the older cells are small and far apart.

#### *b. Relation of cells to lacunae*

In all of the preparations, fixed in alcohol and carefully passed through the steps of the technique outlined above, the cells completely fill the lacunae. In preparations which were carelessly handled or allowed to become dry at any time the cells were retracted from the bony wall. It appears therefore that the term lacunae has no significance other than a place occupied by a tissue cell. Thus in any tissue when a cell is removed a pocket is left which might be called a lacunae with the same degree of correctness as in bone except that due to the plasticity of most tissues the space would collapse.

#### *c. Protoplasmic processes of bone cells*

Not only do the cell bodies completely fill the so-called lacunae but the cell processes fill the so-called canaliculi also. This is especially apparent in figures 2, 5 and 6. In older bones these communicating processes are much finer but there is no indication that they are retracted. In young cells the processes are short and broad at the base and the protoplasmic continuity is unmistakable. The protoplasmic continuity and anastomosis is also shown in older bone (figs. 3 and 4). These figures further show large protoplasmic masses destitute of a nucleus, connected to the cell body by a narrow process. In older bone such masses are rare; but in their places one finds slight enlargements at the points where the anastomosis of several processes occur (figs. 1 and 3). Figure 1, which is a drawing from the

ethmoid bone of a dog, shows an unusual anastomosis between the protoplasmic processes. It appears all the more unusual because they are represented as seen under a shifting focus. Normally many of the processes do anastomose but end blindly as seen in the remaining figures. These mounts of entire bone also make clear the fact that the processes do not lie in one plane only, but that they radiate in all directions to anastomose with the surrounding cells.

*d. The cytoplasm*

The cell protoplasm is of a finely granular nature. Most preparations show a lightly stained, less granular cytoplasm immediately surrounding the nucleus. External to this is an area containing numerous coarse, highly refractive granules. These large granules become fewer toward the periphery but some of them can be found even in the larger protoplasmic processes (fig. 3). There is no sign of lacunar spaces or empty canaliculi but the granular cytoplasm is everywhere in contact with the clear bony matrix.

*e. The nucleus*

The nucleus is normally of an oval shape and is eccentrically placed. Many of the nuclei are shaped differently but such shapes depend on the stage or type of amitosis which they represent. The nucleus is very rich in chromatin which is grouped into smaller or larger masses and irregularly distributed.

*f. The centrosomes*

Centrosomes can be seen in many of the preparations. Gentian violet is not a specific stain for these structures but very good results are often obtained. Iron hematoxylin is a more specific stain but it can not be used for this purpose because it corrodes and stains bone. Further details including their position will be considered later.

*g. Multiplication of bone cells*

A detailed account of bone cell division will be taken up in a subsequent paper on amitosis. In the present paper we shall only attempt to establish the fact that bone cell multiplication does actually occur. In all preparations,—including nasal bones of dogs, cats, and rabbits; ethmoid and sphenoid of man; parietals of the mouse, rat, rabbit and cat; and the femur of young rats,—some stage of either nuclear or cytoplasmic division or both can be seen. The different stages of nuclear divisions are very common and cytoplasmic division is common in young bone. From a previous statement that bone cells form a syncytium it will be clear that cytoplasmic division is not complete in most cases. Since many of the protoplasmic connections break early it is conceivable that all such connections between two sister cells might break. However I am unable to show such a condition. The constriction however is so real that the process of cell division is complete except for a fine connecting process. In all observed cases of nuclear division the method, without exception, was that of amitosis. The constricting or dividing nucleus showed no signs of chromatin rearrangement and the nuclear wall was complete at all stages.

In the region of newly formed bone, multiplication is rapid and often forms strings of cells (fig. 2). These rows of cells are especially seen along the walls of blood vessels radiating toward the periphery as in the parietal, or lying parallel to the long axis of the bone as in the femur. A number of stages of division are shown in figure 6. In figure 4 several masses of cytoplasm without nuclei are separated from the main cell except for a narrow cytoplasmic connection. The largest of these masses is interesting because it contains a small amount of nuclear material and a faint strand of nucleoplasm extends from the main cell nucleus toward this outlying mass of protoplasm. Most of these masses contain no nuclear material. They seem to be pinched off from the main cell by rapidly forming bone matrix in the region of the constriction. The mass containing the nucleus may have been formed in such a way but carrying with it a portion of the nucleus, or it may be the result of cell division

followed by degeneration. This latter interpretation is in accord with Haour's statement that many bone cells degenerate to form phosphoric acid which is essential for the deposition of bone.

#### LITERATURE AND DISCUSSION OF THE BONE CELL

In the study of bone two types of preparations have been employed in the past, namely, decalcified and ground bone. Embryonic bone has been cut without decalcification but the study of such preparations has apparently failed to contribute anything to the knowledge of bone cells. For the study of the bone cells the ground bone method is of little use except to show where the cells were. The method upon which is based our present knowledge of the bone cell is the decalcification and section method. It is upon this decalcification method that the following accounts are based.

In the textbook of Histology by Lewis and Stöhr, the following statement is made:

Active osteoblasts tend to be cuboidal or columnar, but as bone production ceases they become quite flat. They form bone only along that surface which is applied to the matrix. As the strand of bone grows broader through their activity, it encloses here and there an osteoblast, which thus becomes a bone cell (fig. 72). Apparently bone cells do not divide, and if they produce matrix, thus becoming more widely separated from each other, it is only to a slight extent and in young bones; they are therefore quite inactive. Each bone cell occupies a space in the matrix, called as in cartilage, a lacuna, but unlike the lacunae of cartilage those in bone are connected by numerous delicate canals, the canaliculi. In ordinary specimens the canaliculi are visible only as they enter the lacunae, which are thus made to appear stellate. The matrix around the lacunae resists strong hydrochloric acid which destroys the ordinary matrix, and so may be isolated in the form of 'bone corpuscles.' The 'corpuscles' correspond with the capsules of cartilage, which may be isolated in the same way. The bone cells nearly fill the lacunae and send out very slender processes into the canaliculi. These may anastomose with the processes of neighboring cells, as can be seen in the embryo, but it is doubtful if this condition is retained in the adult. The processes, moreover, are so fine that ordinarily they are invisible.

Jordan makes the following statement:



Both in and between the lamellae are many small ovoid spaces which are partially filled by small flattened cells, the bone cells; these spaces are known as lacunae. From each lacuna minute canals, the canaliculi, radiate in all directions, thus placing the lacuna in open communication with its neighbors, and eventually with the lymph spaces of the central Haversian canal. The branching processes of the bone cells frequently project for a short distance into the canaliculi. These cytoplasmic branches are more numerous in newly formed bone, later they are retracted and the cells become more or less shriveled in appearance.

These two accounts typify in general the accounts given in other texts of Histology. Such views are based on preparations of decalcified bone. The picture by Joseph of a bone cell partly filling a lacuna is taken by most authors as the ideal.

*a. Relation of cells to lacunae and canaliculi*

The view that the bone cells partly fill the lacunae and that the canaliculi are for the most part empty is also held by Schaffer. In his textbook of Histology, he refers to Joseph's picture as a typical example. He says, "The cell almost fills the lacunae and may send processes through the canaliculi to anastomose with neighboring cells." It is interesting, however, to note that in commenting on Virchow's statement that the capsules of bone cells were homologous with those of ordinary connective tissue cells, he remarks, "To this may be added that the enclosing lacunae and canaliculi are to be looked upon as corresponding to the cell-space of that tissue." Ranvier thinks that the canaliculi are hollow tubes. Ch. Robin writes that processes extend into the canaliculi in primitive bone but that in old bone the canaliculi are empty. In Keibel and Mall, Bardeen, whose observations were on embryonic tissue, states, "The endoplasmic units, or bone corpuscles, have branched processes which anastomose freely through the canaliculi with those of neighboring cells." Renault presents results which affirm the theory of protoplasmic continuity. He fixed bone in alcohol and also in osmic acid fumes and decalcified in chromic acid or picric acid. In such preparations he found that most lacunae were filled by the bone cells. He obtained his best results with the operculum of Cyprins which he fixed in alcohol, decalcified

and delaminated. In this way he was able to see the relationship of cells within the lamina. Apparently he got results from the decalcified, delaminated operculum similar to those obtained from the thin bones of man, dog, cat, rabbit, rat and mouse which were subjected to no chemical treatment except alcohol fixation. Retterer realized that acid treatment of bone destroyed the actual structure of the cell. He made various attempts to overcome this. He studied small fragments in glycerine and then compared them with sections properly fixed. He observed that the cells and processes completely fill the cavities in the bone. With his description of the structure of the cell, however, I am not able to agree. He found that the central portion of the cytoplasm is dense containing a large amount of chromatin while the peripheral portion is clearer with some strands of chromidial substance radiating from the central portion. Such pictures are common in total bone preparations which have been scraped or also in chips of bone. However, they are always found at the surface where air and fluids came in direct contact with them or sometimes in cases of improper fixation. Such pictures give the impression of protoplasmic coagulation or shrinkage. In fresh bone examined in normal salt solution or in well preserved cells the picture is always like that shown in figure 3.

#### *b. Multiplication of bone cells*

In Lewis and Stöhr's textbook of Histology we read, "Apparently bone cells do not divide." Haour tells us that bone cells degenerate but he says nothing of their multiplication. Apparently Schaefer did not believe in bone cell division for he writes, "The canaliculi, which are at first short, are afterwards extended by absorption so as to anastomose with those of neighboring lacunae." If cells came from a mother cell but never completely separated then the protoplasmic or canicular connections would be present from the beginning of bone formation. Bonome claims that under certain conditions where a rich supply of blood is present, bone corpuscles may give rise to osteoblasts. Bonome made his observations on regen-

erating bone. Macewen agrees with Bonome on this point. Macewen believes also in the multiplication of osteoblasts but it is difficult to determine whether this proliferation occurs in the osteoblast only or in the bone cell also. According to the following account the actual bone cell does not multiply but has the proliferating potentiality.

"The osteoblast is the embryonic or free form of the bone cell, and once formed, is capable of great and rapid proliferation, it has also the power of producing matrix which becomes calcified . . . . The bone cell has the function of surrounding itself with a calcareous zone, which it controls, under the agency of the trophic nerves. As long as the bone cell remains embryonic, it exhibits the power of proliferation; but when it reaches maturity, it assumes the fixed tissue type and becomes stationary." Thus according to him adult bone cells do not divide but possess the proliferating potentiality which property is made use of by cells bordering on an injury.

### *c. Discussion*

It may be hard to comprehend how bone cells enclosed in a prison wall of bone can multiply. It is still more difficult to interpret the many stages of nuclear and cytoplasmic cleavage as any other thing than cell multiplication. When we consider however that young bone is quite soft and pliable and not so extremely different from other dense tissues, except that some lime salts are deposited in it, it will become apparent that cell division and expansion is not as impossible as it at first seemed. The fact that cell bodies are further apart in the old than in the young bone adds to the evidence that a change or movement, such as occurs in growing tissue, must take place. It might be argued that the tissue which I have described as young bone is not bone at all but only unossified matrix. To this we must reply that these preparations conform to mechanical tests for bone. The staining specificity also supports this view. Gentian violet, which stains most tissues intensely does not stain bone at all or only very slightly. All of the illustrations were taken from parts of the preparations where, according to these methods,

bone was present. Thus in figure 2, as in all other figures, the matrix between the cells and processes was unstained just as in figure 1 which is bone from an adult animal. This point is still further established by the fact that toward the periphery of young parietal bones the cells were close together but the matrix which surrounded them was stained so deeply that they were recognizable only with difficulty. Most of these cells are more rounded and at least partly surrounded by uncalcified matrix. Macewen's observations apparently were much like the above except that he considered the cell an osteoblast as long as it was dividing. If this be the case he failed to recognize that ossification set in before proliferation of cells ceased.

The above observations of bone cell division are, however, not entirely new. They are only an extended confirmation of the observations of Nowikoff in the bone of the new-born mouse. He states definitely that bone cells completely surrounded by matrix really divide. He gives two figures on page 369 of his article. Figure 'a' is a very good picture of both cytoplasmic structure and nuclear division but figure 'b' shows signs of cytoplasmic coagulation. It corresponds to Retterer's description of the cytoplasm. The reason for these two different pictures is without doubt due to the fixation. The former was fixed in alcohol while the latter was fixed in sublimate.

#### FUNCTIONAL CONSIDERATION

The foregoing description and discussion of structural details and relationships lead to the question of function. No attempt is made here to answer this important question of bone cell function, however, it is important that certain observations should be reviewed which are suggestive in this connection.

Among earlier writers nothing is said regarding the function of bone cells, these cells are rather regarded as functionless, senile osteoblasts. This view is held by Gegenbaur, Kolliker and Schaffer. They hold that the osteoblast secretes the matrix. Waldeyer and Retterer describe the matrix as a differentiation of the peripheral protoplasm of the osteoblast. Lewis and Stöhr believe that the osteoblast is the active bone former, but they

also attribute a possible function to the bone cell in the following statement: "Apparently bone cells do not divide, and if they produce matrix, thus becoming more widely separated from each other, it is only to a slight extent and in young bones; they are therefore quite inactive."

As early as 1873, Z. G. Strelzoff showed that under certain conditions slight interstitial growth may take place. Jean Haour expressed the view that osteoblasts are not the active bone formers, that they together with the fibroblasts produce a hyaline matrix; but that the engulfed bone cells are concerned with the bone formation. He claims that many of these bone cells degenerate and liberate phosphoric acid which has a special affinity for calcium. Haour states that Gardner has shown that certain highly refractive granules in bone cells liquefy and that this liquid is directly concerned in the process of ossification.

In 1901 Fujinami in his paper on 'Tissue changes in healing of Bone Fractures' writes, "In sparrows I saw at times a nucleus in the middle of an elongated spicule of ossific ground-substance, both sides of which were closely lined by osteoblasts. The nucleus, whose shape very nearly resembled that of the spicule, showed a rim of finely granular protoplasm, which, without a sharp boundry, gradually passed over in all directions, especially in the long axis, into the ground substance." He also found in his stained preparations that the osteoblasts were differentiated very sharply from the spicule on which they were lying. He implies in this description that the bone cell rather than the osteoblast is the active bone former.

That the bone cell is not entirely inactive is certainly brought out by many of these observations. Its exact relation to bone formation however, is differently expressed by the observers. Haour attributes bone formation to the degenerating cell. Gardner attributes it to the granules in the cell. According to Fujinami the peripheral protoplasm gradually passes over into bone.

Many of the observations presented in this article affirm the position that the bone cells are actively concerned in bone formation. What the exact relationship between matrix and bone cells is I am unable to say at the present time.

The structures which I have observed that indicate that bone cells are active in bone production may be enumerated as follows:

1. Bone cells are much farther apart in old than in young bone.
2. Bone cells as a rule are somewhat smaller in old than in young bone.
3. The large protoplasmic masses which lie in the matrix of young bone are almost absent in old bone. This may either indicate that they are used up in bone formation or it may have some bearing on Haour's degeneration theory.
4. The fact of cell multiplication certainly indicates cell activity.

#### SUMMARY

1. Bone cells are elongated or rounded bodies with protoplasmic processes radiating in all directions. The cells with their processes completely fill the so-called lacunae and canaliculi. Many of the processes anastomose with similar processes of neighboring cells.
2. The cell protoplasm is finely granular with highly refractive or deeply stained granules distributed through the more peripheral portion.
3. The size of the cell depends on the state of proliferation and on the age of the cell. Old cells are usually a little smaller than young cells.
4. Bone cells are further apart in old than in young bone.
5. The large protoplasmic masses, lying in the bony matrix of young bone and which are connected to the main cell by narrow strands of protoplasm have almost entirely disappeared in old bone.
6. The nucleus is rich in chromatin which is grouped in small masses and irregularly distributed.
7. Bone cells multiply. The multiplication is rapid in young bone and slower in old bone, but division is always by amitosis.
8. Bone cells are not senile, functionless osteoblasts but observations indicate that they are active cells which are in some way related to bone production.

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**PLATE**



## PLATE 1

### EXPLANATION OF FIGURES

Camera lucida drawings from entire bone which was fixed in 95 per cent alcohol and stained in gentian violet. The original magnification as indicated for each figure was reduced in reproduction two fifths.

1 Relationship of cells in the ethmoid bone of an adult dog. The protoplasmic processes show an unusual degree of anastomosis. This is partly due to the fact that the anastomosis is indicated as seen under a slightly shifting focus.  $\times 567$ .

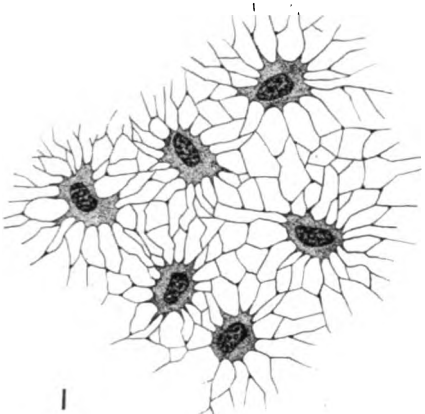
2 Group of young cells near the periphery of parietal bone from a 15-day old rat. Cells arranged in rows and show signs of rapid division. Some nuclei are still in the process of division.  $\times 720$ .

3 Group of cells from the center of a 15 day old rat parietal. Centrosomes can be seen in a number of these cells.  $\times 720$ .

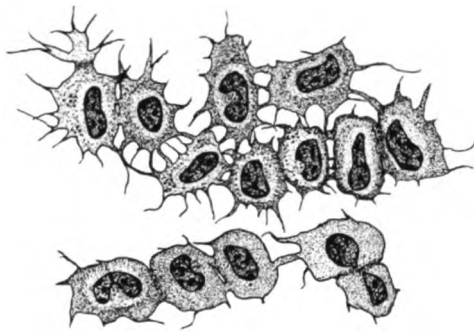
4 Three cells from the center of parietal bone in a one day old rat. The striking features of this picture are the large protoplasmic masses almost separated from the main cell bodies. The mass to the left contains nuclear fragments.  $\times 720$ .

5 Cells from the parietal of a new born rat. Note broad protoplasmic bands connecting the different cells.  $\times 720$ .

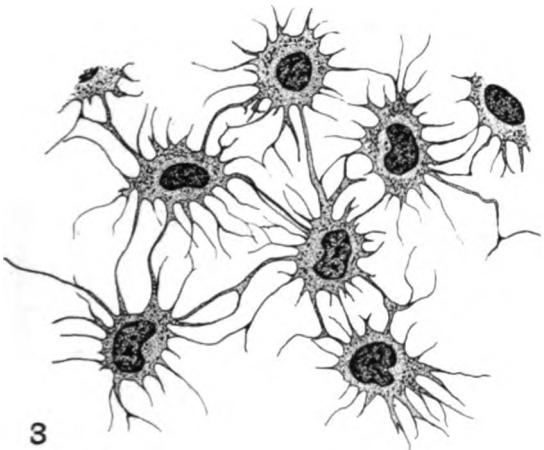
6 Cells from the peripheral portion of a 15 day old rat parietal. These cells are younger than those in figure 2. They have small protoplasmic processes and resemble osteoblasts. The matrix is slightly tinted by the stain which indicates that not much bone is deposited. Several nuclei are already dividing.  $\times 720$ .



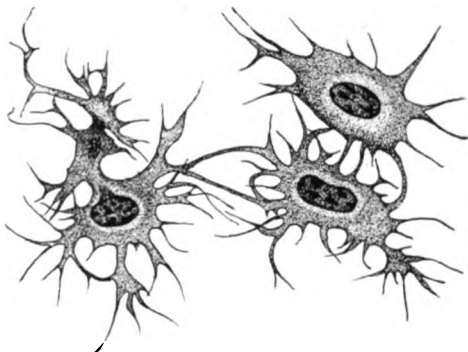
1



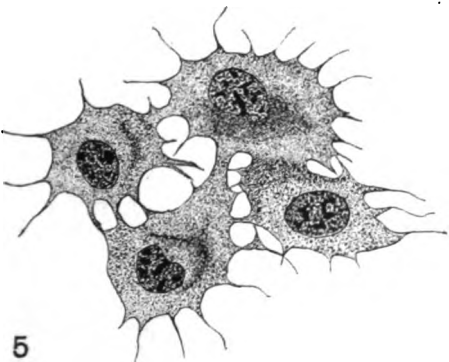
2



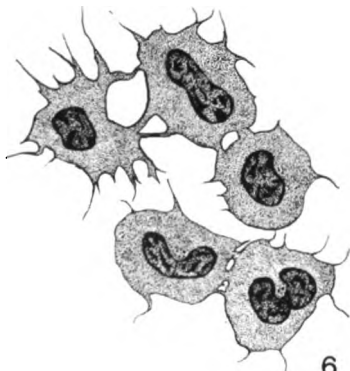
3



4



5



6

Resumen por el autor, John Stephens Latta

**La histogénesis del tejido linfático denso del intestino (*Lepus*):  
Una contribución al conocimiento del desarrollo del tejido  
linfático y de la formación de las células sanguínea.**

El objeto de la presente investigación es determinar con la mayor exactitud posible el origen y desarrollo de los diversos tipos de células sanguíneas alrededor y dentro de los nódulos linfáticos del intestino y sus proximidades, en la región cecal del conejo. Las primeras células libres que aparecen son los pequeños linfocitos, los cuales se desarrollan in situ a expensas de las células del mesenquima. Unos pocos hemoblastos linfoides se desarrollan también por transformación directa de las células mesenquimatosas. Estos dos tipos son aparentemente estados diferentes del crecimiento de una misma célula; los pequeños linfocitos, mediante crecimiento y ligera transformación, se convierten en hemoblastos linfoides, y estos últimos mediante divisiones repetidas se reducen a pequeños linfocitos. La presencia de grandes macrófagos acidófilos que derivan mediante diferenciación ulterior de los hemoblastos linfoides y la de pequeños linfocitos en vías de degeneración indica que el llamado centro germinal es más bien un centro de degeneración que de proliferación. Los eosinófilos de forma binucleada se desarrollan en el tejido conectivo cerca de los nódulos, especialmente en la túnica propia. La abundante eritropoiesis extravascular se encuentra en el conectivo subnodular o internodular, diferenciándose las células eritroblásticas a expensas de los hemoblastos linfoides. El desarrollo de los tres tipos de células sanguíneas parece estar en cierto modo relacionado con la abundancia de capilares. La formación de tejido linfático nodular puede depender de condiciones nutritivas, suministradas por la sangre. La diferenciación ulterior de los hemoblastos linfoides depende probablemente de la proximidad de asociación con los vasos sanguíneos, la lentitud de la corriente y el espesor de las paredes vasculares; la relación muy íntima de los hemoblastos y la corriente sanguínea produce eritropoiesis, y una conexión más remota da lugar a granulopoesis.

Translation by José F. Nonidez  
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# THE HISTOGENESIS OF DENSE LYMPHATIC TISSUE OF THE INTESTINE (LEPUS): A CONTRIBUTION TO THE KNOWLEDGE OF THE DEVELOPMENT OF LYMPHATIC TISSUE AND BLOOD-CELL FORMATION

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FOUR PLATES (SIXTEEN FIGURES)

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## INTRODUCTION

The origin, development, and fate of the various cellular elements of the blood, their relation to the loose connective tissue of the body, and the conditions associated with, or causing their production, are still unsettled and debatable questions despite the vast amount of previous investigation of hematological problems.

Some of the most perplexing of these problems arise in discussion of the production of lymphatic tissue, or lymphopoiesis, as found in various places in the body, more particularly in the tonsils of the mouth and intestine. Just what are the conditions which bring about or are associated with the formation of lymphatic tissue? Are these conditions controlling its formation the same, wherever it occurs, e.g., in the tonsils as in the lymphatic nodes? Or are there different conditions, the presence of any one of which may initiate and control lymphopoiesis? Does lymphopoiesis, wherever occurring in the body, always serve the

same purpose? Is the function of the tonsils of the mouth and intestine the same as that of the lymphatic nodes? These questions and others, in view of the evidence set forth in previous investigation, make the interpretation of the lymphatic tissue difficult.

#### HISTORICAL

This confusion, regarding questions of lymphopoietic processes is added to, in the study of the histogenesis of tonsillar tissue, by the close relationship existing between the lymphatic tissue of the tonsils and the overlying epithelium. This relationship in fact, when the development of the tonsillar lymphatic tissue first became a matter of investigation, led to the conclusion by some authors that the lymphocytes in these regions were of epithelial origin.

This belief was most vigorously defended by Retterer, who, in a series of papers ('91, '92, '93, '09, '13) on the development of the various tonsils, in each case declared the lymphocytes of these structures were derived by downgrowths from the overlying epithelium. Von Davidoff ('86-'87), working on the formation of lymphatic nodules in the jejunum of man and the appendix of the guinea-pig, and Klaatsch ('92), studying the histogenesis of Peyer's patch in the *Echidna*, are among those who, like Retterer, thought the lymphocytes thus formed were of epithelial origin.

The majority of authors concerned with problems relative to the histogenesis of tonsillar lymphatic tissue, however, disagreed with these conclusions. Chief among these was Stöhr, who, in studying practically the same material as Retterer did, arrived at very different conclusions. He found the epithelium remaining inactive in the formation of the tonsillar lymphatic tissue, the elements of which were derived from the mesenchyme of the tunica propria. Others agreeing with Stöhr in ascribing to these cells in tonsillar structures a mesenchymal origin include Flesch ('88), Zwarykin ('89), Tomarkin ('93), Kückenmeister ('95), and, more recently, Mollier ('13) and Hartman ('14).

This diversity of opinion among earlier investigators as to the development of tonsillar lymphatic tissue (i.e., in the intestine) and the practical omission of such study by more recent investigators, together with the great amount of doubt and uncertainty concerning questions of the origin and development of lymphatic tissue in general, seem to warrant further study of the intestinal tonsillar tissue (Peyer's patch and the appendix) in an attempt at some interpretation of these structures.

#### MATERIALS

As material for the pursuit of such investigation, the rabbit was considered as well suited, chiefly because of the great concentration and remarkable development of lymphatic tissue in Peyer's patch in the lower portion of the ileum, causing an abrupt enlargement in it at its point of junction with the caecum, the sacculus rotundus, or iliac tonsil (Muthmann, '13).

Also, by use of this form, abundant material was easily obtainable in all stages of the development of these intestinal tonsillar structures. It was found after short study that in newborn animals or earlier stages that there were no indications of the formation of lymphatic nodules either in the region of Peyer's patch or the appendix, so no embryonic material was collected. Females were secured, either in late pregnancy or just following the birth of the litter, and the young rabbits removed from the litter at desired intervals. The first indications of nodular lymphatic tissue were found to be at an age of from two to three days, free cells beginning to be massed in the location of future nodules. From this time growth and differentiation proceed at a rapid rate, until at an age varying from five to seven weeks the nodules have assumed a structure like that found in the adult individual (except for size).

During this time of very rapid development, stages were secured at very frequent intervals to be sure to get an uninterrupted developmental picture. In practically all cases two or three individuals of the same age were secured, to correct any deviation which might occur in any single instance. Animals were secured at daily intervals, and sometimes half-day intervals,

ranging from newborn to individuals of fifty-six days postpartum; that is, to a time after the intestinal tonsils had assumed an adult structure. Tissues from older individuals at less frequent intervals, were also obtained, up to an age of about two and one-half months.

Helly's fluid (Zenker-formalin) was used almost exclusively as a fixing reagent, as did most recent investigators of hematological problems. With it, in the writer's experience, is given the best general fixation, without in any way interfering with desired subsequent staining with the compound blood stains. In a few instances a fixer suggested by Downey ('15) was used. This was a 0.9 per cent normal salt solution saturated with  $\text{HgCl}_2$ , to which 10 per cent of formalin was added at the time of using. This gave results apparently equally as good as Helly's, but there being no advantages held over the latter, it was seldom used.

The tissues were all imbedded in paraffin and cut in sections from 5 to 7  $\mu$  in thickness.

The stain used most extensively in this study was Hasting's modification of the Nochts-Romanowsky blood stain. Wright's blood stain was also used to some extent, but the Hastings-Nochts combination was equally as selective as Wright's for cells of the blood series, and gave a more brilliant, intense and a sharper nuclear stain. The sections were first stained six to ten minutes in the concentrated stain, and then removed, without washing, to a solution of the stain diluted one-half with distilled water for ten to fifteen minutes. The sections were then differentiated and dehydrated in 95 per cent and absolute alcohol, cleared in several changes of neutral xylene, and mounted in neutral xylene damar.

A combination composed of a 1 per cent solution of eosin in methylic alcohol, followed by a weakly alkaline, aqueous solution of methylene blue (Gage, '17), was found to be almost as selective a stain for blood cells as Hastings-Nochts, and to give a still more brilliant, sharp picture. Therefore, this stain was quite extensively used. The sections were carried immediately from 95 per cent alcohol into the 1 per cent methyl alco-

holic eosin, washed in water, then carried into the methylene-blue solution, after which they were differentiated, dehydrated, cleared, and mounted as for the Hastings-Nochts stain.

Another stain, proving of no little value in bringing out certain specific features, was a mixture composed of equal parts of 1 per cent aqueous solutions of methyl green and pyronin (after Pappenheim). After staining in this solution for ten minutes, the sections were washed in water, and carried immediately into a 1 per cent solution of resorcin in absolute alcohol for differentiation and dehydration, cleared in xylene, and mounted in xylene damar.

For the purpose of sharply differentiating the reticular tissue from the free cells of the lymphatic tissue, Mallory's connective-tissue stain was used, after first mordanting the sections for a few minutes in picro-acetoformalin.

Other special methods were used at various times to bring out or make clear certain specific features of these structures. These methods will be discussed as the features made clear by their use are spoken of.

In the study of the histogenesis of the elements of the connective tissue (tunica propria and submucosa) in the region of the formation of the intestinal tonsils, it is noted that, coincident with the formation of lymphatic tissue, other cellular elements of the blood may be developing also.

It has long been a matter of common knowledge that, in the connective tissue of the wall of the digestive tract, varying numbers of cells are found, in the cytoplasm of which are granules of either an eosinophilic or basophilic character. As has been stated, the granules of basophilic character are readily soluble in water and consequently were not found in any of the preparations used, being dissolved out in the course of the preparation. But the cells with eosinophilic granules are found present, often in extraordinary abundance, in the connective tissue in the neighborhood of the lymphatic nodules, more particularly in the tunica propria (figs. 9, 10, 11, 14, 16).

Also, at certain stages in the development of the tonsillar lymphatic tissue, there may be found, in apparent association



with its formation in the subnodular and internodular connective tissue, groups of cells in developmental stage of the formation of erythrocytes (erythroplastids). These erythroblastic cell groups are not of constant appearance, and, when present, vary greatly in number, sometimes there being but a few erythroblastic cells scattered about in the connective tissue and, again, large masses of them, or foci, were found. These erythropoietic foci were found in largest extent in the region of the iliac tonsil (Peyer's patch), but in a few cases erythropoiesis was noted to be occurring to a very limited extent in the connective tissue of the wall of the appendix (figs. 7, 14, 15).

This phenomenon of erythropoiesis is not confined to any exact, definite period of development, but it does, however, occur most frequently and in greatest amount in animals from two to six weeks after birth. Even though, as before stated, the presence of erythropoietic centers is not of absolute constancy, they are found present so frequently (and in such number) as to render improbable the thought that they arise because of some particular pathological condition in each case.

It is apparent, therefore, that in the study of the histogenesis of elements of the connective tissue in the region of the intestinal tonsils of the rabbit, one is confronted with the problems concerned with the formation of all the types of blood cells, which may be divided into three phases, according to the character of the resulting cells: 1) lymphopoiesis, the development of the components of the lymphatic tissue, the lymphocytes and reticulum; 2) the development of cells containing in their cytoplasm acidophilic granules, or granulopoiesis, and, 3) the development of red blood cells, or erythropoiesis. The origin, development, and the fate of these three different types of cells, and conditions causing and controlling their production, as well as interrelations which might exist between the three phases of development, are questions for which this material gives opportunity for study.

## LYMPHOPOIESIS

The most extensive formation of blood cells in the region of the intestinal tonsils is, of course, that of lymphocytes. The free cells, or lymphocytes, which make up the bulk of the nodular tissue of the tonsils have been classed by hematologists into two groups, 1) small, round cells, with a round or slightly oval nucleus, the heavy chromatin particles of which are arranged just within the nuclear membrane so as to appear somewhat as the spokes of a wheel, inside a definite nuclear membrane, covering which is a thin rim of densely basophilic cytoplasm, the small lymphocytes, and, 2) comparatively much larger cells potentially round, but evidently possessing some ameboid tendencies, with a clear vesicular nucleus, round or oval in form, containing a very scanty amount of chromatin, lying for the most part just within the nuclear membrane, and usually a prominent nucleolus. Surrounding the nucleus is a varying amount of densely basophilic cytoplasm. These are the so-called large lymphocytes, or lymphoid hemoblasts.

The relationship between these two types of cells, and their potentialities, distinction as permanent cell types, etc., have long been perplexing and debatable questions. The majority of present-day hematologists seem to consider the small lymphocyte as the more distinct permanent cell type, with no potentialities other than the production of others of its kind, while they ascribe to the other type of cell a more blastic nature, with potentialities of differentiation into cells of any of the three types, given the correct environmental conditions.

The question of the origin of these lymphocytes, especially as occurring in the tonsils, has long been a matter of much controversy. The somewhat spirited debate between Retterer and Stöhr, already mentioned, may be recalled. Retterer insisting upon an epithelial origin for the lymphocytes, and Stöhr equally sure that the epithelium remained passive in their formation, the cells of origin being of the mesenchyme. These and others of the earlier authors did not recognize the two types of lymphocytes. The recognition of these two types complicates the question of their origin still further.

The large (clear-nucleated) cells of the lymphatic nodules, lymphoid hemoblasts, have been described under various names by investigators of problems of blood-cell formation. This type of cell is identical, morphologically at least, with the free wandering cells of the loose connective tissue, the so-called primäre Wanderzellen of Saxer ('96), the primitive large lymphocyte or wandering cell of Maximow and others, hemogonia of Mollier ('13), lymphoblast of Naegeli, mesameboid of Minot, lymphoid hemoblast or hemocytoblast of Danchakoff, etc.

The term, proposed by Danchakoff, seems to be the most descriptive of this type of cell, for, as she pointed out when suggesting this term, it is essentially of a lymphoid nature, and it is quite generally accepted (monophyletic theory) that this type of cell possesses potentialities capable of transforming into any of the many different cellular elements of the blood under favorable environmental conditions. Although environmental conditions are normally such, in lymphatic nodules, that this type of cell remains as a cell of the lymphocyte series, it is believed to retain this potentiality of further differentiation into other types of blood cells (as in one instance, noted by the writer, in which all the lymphoid hemoblasts of a lymphatic nodule of Peyer's patch in a twelve-day-old rabbit had transformed under pathological change of environmental conditions into eosinophilic granular leucocytes). The term, lymphoid hemoblast, will in this article refer to this type of cell.

Hematologists are practically united in ascribing to this cell an origin from the fixed cells of the body mesenchyme, or embryonic connective tissue, Danchakoff maintaining that they may also derived from cells of the vascular endothelium.

But the origin of the small lymphocytes, their potentialities, and relation with the lymphoid hemoblasts are yet much debated questions. Macimow thought the small lymphocytes and lymphoid hemoblasts or large lymphocytes were one and the same cell in different growth stages. Badertscher ('15) found, in the developing thymus, that the small lymphocytes were derived by repeated divisions of 'large lymphocytes,' but is not sure whether the small lymphocytes may again grow into the other type or not.

Downey and Weidenreich ('12) found, in lymphatic nodes, the small lymphocytes were developed from the large reticulum cells of the node. These small lymphocytes, they thought, by growth became 'large lymphocytes.' Danchakoff ('16) found that small lymphocytes in the spleen arose by differentiation of dwarfed lymphoid hemocytoblasts, which had arisen because of intense proliferation and poor nutrition of normal lymphoid hemocytoblasts. The small lymphocyte she considered a distinct, stable cell form, incapable of growth into the lymphoid hemoblast.

Aside from this question of relationship in connection with the study of the formation of lymphatic tissue, in the lymphatic nodes and the tonsils, the question still remains as to whether these lymphocytes arise in situ, wander in from the mesenchyme in other places, or are carried in and dropped by the blood vessels.

Those studying the development of lymphatic nodes are not united on this question. Gulland ('94) thought the first lymphocytes to appear in developing nodes were filtered from the blood stream. Saxer ('96) considered that they arose in situ from the 'primäre Wanderzellen' of the mesenchyme. Sabin ('05) also favored this view, although she considered the evidence at hand insufficient to definitely determine the origin of the lymphocytes. Downey and Weidenreich, as before stated, also thought they arose in situ by differentiation of the reticulum cells of the node.

Hartmann ('14), who studied the development of the intestinal tonsillar tissue of the rabbit, considered that the lymphoid hemoblasts, at least, arose in situ by differentiation of mesenchymal cells, but he did not state any definite conclusions as to the origin of the small lymphocytes.

A study of the connective tissue in the region of the intestinal tonsils of the newborn rabbit, as stated before, revealed no traces of nodular lymphatic tissue. First evidences of future nodules are seen at an age of two to two and one-half days, at which time there is an apparent heaping up or condensation of mesenchymal or embryonic connective-tissue cells of the mucous membrane underneath the epithelium between the bases of the villi. These condensations are due to the appearance of free cells in the meshes of the mesenchymal reticulum. The free cells are, for

the most part, small round cells, with a round nucleus, fairly dense with chromatin, surrounded by a thin rim of densely basophilic cytoplasm. These are considered to be true small lymphocytes or late developing stages of the same, for they are morphologically identical, except that the chromatin particles are not always so heavy in the free mesenchymal cells as in the typical adult small lymphocyte. Many developmental stages are found, showing every possible transition between the fixed mesenchymal cell to the free small lymphocyte (figs. 1 and 2).

The small lymphocytes vary somewhat in size, due, doubtless, to varying sizes of the mesenchymal cells from which they develop. Developmental stages between fixed mesenchymal cells and lymphoid hemoblasts are only occasionally seen. It is exceedingly difficult to classify some of the free cells, as many intermediate stages between typical small lymphocytes and lymphoid hemoblasts are found. Typical adult lymphoid hemoblasts are very rarely found in the lymphatic tissue during the first week of postfetal life.

The small lymphocytes increase in number quite rapidly, both by transformation of mesenchymal cells and by proliferation of those already formed. Mitotic figures in small lymphocytes are numerous. During the first few days of postfetal life, a few mitotic figures can be seen in the ordinary mesenchymal cells also.

The larger lymphoid hemoblasts do not begin to be present in any quantity until some time during the second week of postfetal life. When they become present to any extent, frequent mitoses may be seen to occur in them. It seems reasonable to assume that, if these divisions are repeated at frequent enough intervals, the size of the resulting cells would be much decreased; i.e., to the size of a small lymphocyte.

The small lymphocytes, rather than the lymphoid hemoblast, seem here to be the first type to develop. Their origin seems threefold; those first appearing developing only by direct transformation from mesenchymal cells and by proliferation of those already formed, and later on appearing also as a result of repeated rapid divisions of lymphoid hemoblasts.

It is also apparent that there are also two sources of origin for the lymphoid hemoblasts; first, by direct transformation of larger mesenchymal cells and, secondly, by growth of small and medium-sized lymphocytes, which have previously been formed from the mesenchyme.

These facts are added evidence to the view held by several authors (Maximow, Weidenreich, and Downey, etc.) that small and large lymphocytes (lymphoid hemoblasts) are not distinct cell forms, but merely different growth stages of the same cell, there being here a growth cycle, the small lymphocytes, by growth, becoming lymphoid hemoblasts, and the latter, by repeated divisions, forming small lymphocytes.

Because of the characteristic wheel-like arrangement of nuclear material in the small lymphocytes (which arrangement is not as characteristic of small lymphocytes in the rabbit as in some other forms), one is inclined to regard them as a definite cell form. The difference in the appearance of the nuclei of the two forms is, however, partially explained by the crowding together of the nuclear material into smaller area, which would also tend to obscure a nucleolus, if such were present.

But in case it is accepted that small lymphocytes and large lymphoid hemoblasts are different growth stages of the same cell form, it must be remembered that the cell as found in the small lymphocyte stage possesses different potentialities than when in the lymphoid hemoblast stage. The small lymphocyte may produce, by division, others of its kind, or by growth, a lymphoid hemoblast, which is the limit of its potentialities. It cannot possess the potentiality of producing other cells of the blood series without first development and growth into a lymphoid hemoblast (i.e., a small lymphocyte will never produce directly a granulocyte or erythroblast without, first, growth into a lymphoid hemoblast).

In studying the relations causing or associated with the formation of lymphatic tissue, it is found that this tissue always develops in places where there are rich lymphatic plexuses, and also in close relationship with the blood capillaries. These relationships were noted by Gulland ('94), Saxer ('95), and Sabin ('04) in developing lymphatic nodes. Hartmann ('14) decided

that the submucosal lymphatics of the intestine do not serve primarily in the transportation of chyle, but are, in a certain sense, related to the formation of follicles. As evidence of this he finds the first large groups of lymphocytes arranged about vessels, "as in lymph glands, and the tonsils of the mouth."

In the present study of the region of the intestinal tonsils, it was found that in the newborn animals and late fetuses before there were any evidences of lymphatic tissue and before absorption of food had started, that lymph vessels had formed quite extensively in the submucosa, making quite a rich submucosal plexus, such as the plexuses Gulland, Sabin, and Saxen found in regions of developing lymphatic nodes. This plexus connects with the retroperitoneal sac and through it with the thoracic duct and the venous system by means of lymphatics of the mesentery. (Heuer, '09; Sabin, '14). When the lacteals and the mucosal plexus form, they become connected with the submucosal plexus, the latter then becoming a part of the system for the transportation of chyle. None of the lymphatic vessels, then, which are found in the wall of the intestine are connected with any entering vessels, the lymph flow being all directed away from the intestine. Consequently, any lymphocytes which may be found in the lymph vessels in the intestine wall have probably entered them from the surrounding lymphatic tissue rather than have been carried in by the lymph stream. Thus the theory that lymphocytes of the intestinal tonsillar lymphatic tissue have been carried in and left by the lymph stream seems highly improbable.

It is noted, however, that there is also a close relationship between the blood capillaries of the mucosa and submucosa and the developing lymphatic tissue of the intestine. The first lymphocytes to appear, however, bear very little or no definite relation to the blood vessels, and only after they become present in considerable numbers do they appear to gather in clumps about the blood capillaries. This seems to the writer to indicate that the part played by the blood vessels should be considered as a nutritive one rather than a source of lymphocytes, as was thought by Gulland.

One factor apparently common to the formation of lymphatic tissue anywhere is that of an extensive supply of lymph vessels or lymph plexuses. It seems probable, therefore, that there is some influence exerted by the lymph or lymph vessels on the surrounding mesenchymal or loose connective tissue to induce lymphopoiesis. These lymph plexuses are always found in places where the blood supply is also very extensive. This very rich blood supply affords excellent nutritive conditions for the growth and multiplication of lymphocytes after the lymphocytopoietic reaction has been initiated by the lymph plasma or the lymph vessels.

Coincident with the differentiation of mesenchymal cells of the mucosa into free lymphocytes, there occurs a formation by other cells of the mesenchymal reticulum of connective tissue. This is at first a richly cellular, embryonic connective tissue. As the formation and proliferation of lymphocytes proceeds, the free cells separate the fibers of the connective tissue so that a branching, reticular network of fibers, reticular tissue, is formed, a skeletal framework, in the meshes of which the lymphocytes proliferate.

The first masses of cells formed in the region of the future Peyer's patch and of the nodular tissue of the appendix, composed mainly of small lymphocytes and transitional stages between them and mesenchymal cells or those of the embryonic connective tissue, bear little resemblance to adult lymphatic nodules (fig. 1). Lymphoid hemoblasts appear first to any extent at an age of eight days or later. Until this time the formation of most of the free cells has been by differentiation from mesenchymal cells. Many transitional stages between mesenchymal cells and free lymphocytes were seen in all preparations of tissues studied up to this time in development. At about the ninth or tenth day after birth mitotic figures begin to appear with some frequency, especially in the small lymphocytes, and the masses of cells at the same time begin to assume a more definite nodular shape. Lymphoid hemoblasts constantly increase in number, both by differentiation from mesenchymal cells and by growth from small lymphocytes. These lymphoid hemoblasts



assume no definite position in the nodules, but are found scattered throughout (figs. 3 and 4).

As the number of lymphocytes in a nodular mass increases, the connective tissue surrounding it tends to become pushed back and piled up or condensed apparently by outward pressure of the increasing number of lymphocytes, and the fibers and connective-tissue cells (fibroblasts) tend to assume a circular or concentric arrangement about the mass of lymphocytes, thus giving rise to a fairly dense sheath or capsule, which gives the nodule more definite limits and outline.

This sheath or capsule is by no means as complete and definite a structure as is found, for example, in the capsule of lymphatic nodes, but is rather a physical expression of the proliferation of lymphocytes in regions of unusually rich vascularity. Lymphocytes are always found to some extent among and outside of the connective-tissue fibers of the sheath, being by no means strictly confined within it.

In the process of the proliferation of lymphocytes and the formation of the connective-tissue sheath, reticular fibers and cells are gradually forced outward from the center of the mass, so that in later stages of nodular development few or no fibers or reticular cells can be demonstrated except near the periphery of the nodule.

Although the nodular sheath does not form an impassable barrier for the passage of lymphocytes, its presence and the fact that the reticulum is so much closer meshed at the periphery tend to inhibit the spreading of lymphocytes which are rapidly increasing in number. This inhibition causes the lymphocytes to become more closely packed at the periphery of the nodule than in the center. Thus the center of the nodule, because of the lesser number of cells there, appears lighter than at the periphery. This lighter appearance of the center of the nodule was noted by Flemming ('85) and named by him the 'Keimzentrum,' or 'germinal center,' for he considered this lighter central area a center of proliferation of lymphocytes. Later investigators have followed Flemming in calling this area the germinal center of the

nodule, a term which, as will be shown later, must certainly be considered as non-descriptive of existing conditions.

Flemming, in studying the structure of nodular lymphatic tissue found the lighter appearance in the center of the nodules was due largely to the fact that the cells making up that center were mostly those containing comparatively large, rather lightly staining nuclei with a relatively large amount of cytoplasm about them (germinal center cells, lymphoid hemoblasts), so that the nuclei were farther apart, while, on the other hand, those cells at the periphery were those with much smaller, more dense nuclei, with a smaller amount of cytoplasm surrounding them (small lymphocytes), so that when seen en masse the more closely packed, dense nuclei gave a darker appearance to the outer portions of the nodule than that of the central portion.

Although the evidences of proliferation, mitotic figures, were not confined strictly to the lighter colored central area, he found them occurring most frequently there, and not all in the dark, peripheral zone. Therefore, he called the lighter central area of the nodule, the germinal center. Other authors holding to this theory advanced by Flemming include Baum u. Hille, Baumgartner and Ribbert, Saxer, etc.

Others believed the germinal center was an expression of the rapid proliferation of lymphocytes, which proliferation, however, was scattered throughout the lymphatic tissue (Weidenreich and Downey, '12, also Maximow, Mollier, et al.).

Several authors noted the preponderance of cells of the lymphoid hemoblast variety in the lighter colored center, and some, consequently, named them 'germinal center' cells. Hartman ('14) found the germinal centers of the nodules of the intestinal tonsils to be due to this fact.

In the present investigation of the tonsillar lymphatic tissue of the rabbit it was found that the lightly staining central area in the nodules appeared at no very definite time; this depending, apparently, somewhat upon conditions which varied according to the individual. However, there is usually some little evidence of the lighter colored center at about the age of two weeks, but

it does not become well marked till an age of twenty-one days or older. Invariably the nodules of the appendix are further advanced in this respect than are those of Peyer's patch. At an age of twenty-four days, however, the lighter area is very definite both in nodules of the appendix and of Peyer's patch.

A study of all developmental stages and of adult nodules clearly indicates that these so-called 'germinal centers' are not centers of proliferation. In young animals mitotic figures are numerous in both the lymphoid hemoblasts and the small lymphocytes, but these mitotic figures are not confined to the center of the nodule, being apparently scattered throughout the nodule. In fact, in older stages, if anything, they seem more abundant out near the periphery of the nodule than in its center (figs. 3 and 4).

Mitotic figures are surely not confined to large or medium-sized lymphoid hemoblasts, for mitoses are very frequent in the small lymphocytes.

The grouping of lymphoid hemoblasts or germinal center cells in the center of the nodule to produce its lighter appearance could not be seen. In nodules in which the so-called 'germinal center' was most prominent, the lymphoid hemoblasts and the small lymphocytes were scattered equally throughout the nodule. At no time was there noted a special grouping of lymphoid hemoblasts in the center of the nodule or of small lymphocytes about the periphery. The proportion of small lymphocytes to lymphoid hemoblasts is great, but that proportion is the same throughout the nodule.

The so-called germinal center, then, should not, as that name would indicate, be considered a center of proliferation of the lymphocytes. Its lighter appearance is due merely to a different concentration of lymphocytes here than at the periphery of the nodule. The reason for the concentration of lymphocytes at the periphery is the purely mechanical one of the lymphocytes being held there in greater concentration by the denser reticular network and the surrounding capsule of connective tissue. In any mass of proliferating cells the natural tendency is to spread outward, which tendency is, in this case, checked somewhat by the

surrounding connective tissue causing the cells to pile up at the periphery.

Careful examination of the central portions of the nodules reveals the fact that the different concentration of cells is not alone responsible for the lighter appearance of the center. There are found among the elements making up a nodule, certain large cells, whose abundant cytoplasm is acidophilic in character, surrounding a fairly large nucleus, which also has a tendency to react to acid stains or stains only weakly basophilic. Some of these large acidophile cells were observed to contain in their cytoplasm bodies of various sizes and reacting variously to basis stains (fig. 5).

These cells have been quite differently described and interpreted by authors who studied them. Flemming ('85) described cells in the germinal center of lymphatic nodules, the nuclei of which were identical with the 'large lymphocytes,' containing in their cytoplasm various deeply staining bodies, which he designated stainable bodies. The nature of these bodies he did not know.

Downey and Weidenreich ('12) also found in the germinal centers large free cells, derived from cells of the reticulum which possessed phagocytic powers. The phagocytized elements they thought comparable to the stainable bodies of Flemming, which were possibly cellular, particularly nuclear remnants.

Hartmann ('14) also saw large acidophile cells in the nodules of the intestinal tonsils, which did not occur in lymphatic tissue elsewhere in the body. These were large, free, irregular shaped cells, which were derived from cells of the reticulum, with cytoplasmic inclusions. The inclusions he found to be always round or oval, and concluded they must be of a fluid or gelatinous nature. Because they stained a 'braunrosa' color with sudan III he thought them to be of a lipoid nature.

The large acidophile cells found present in the intestinal lymphatic tissue are, morphologically, very similar to the cells described by Flemming, Weidenreich and Downey, and Hartmann. The cytoplasmic inclusions certainly do not, however, react as lipoids, as according to Hartmann, but in appearance

and staining reactions are identical with the 'tingible Körper' as described by Flemming.

The nature of the inclusions, if they are such, and the origin, fate, etc., of these acid-staining cells have not been satisfactorily determined. If these deeply staining bodies are foreign bodies, phagocytized by the non-granular acidophile cells, what, then, are the foreign bodies derived from?

If these bodies are inclusions and the cells containing them phagocytic, the cells should then take up particles of colloidal dyes if solutions of the dye are injected into the animal's body.

In order to determine whether the acid-staining cells possessed any phagocytic power or not, solutions of the acid azo colloidal dye, trypan blue, were used. Large quantities of a 5 per cent aqueous solution of the dye were injected into the peritoneal cavity of rabbits. In these instances all the dye was phagocytized by the large free cells in the peritoneal cavity and cells of the subperitoneal connective tissue. In order, then, to get the dye in a position where it could be acted upon by cells in the intestinal lymphatic nodules, operations were performed in which the peritoneal cavity was opened and a 5 per cent aqueous solution of the dye injected directly into the lymphatic tissue of Peyer's patch and the appendix, and the wound sewed up. After twenty-four to forty-eight hours the animals were killed and the tissue fixed in the usual way.

Examination of this tissue revealed the fact that large quantities of the dye had been phagocytized by the acid-staining cells and by the connective-tissue cells outside the nodules, the lymphocytes being free of it, except immediately about the point of injection, where they were diffusely stained, the cells at this point being apparently killed by the shock of injection. This shows clearly that these cells possess phagocytic powers and belong to the group of cells called macrophages (Evans) (fig. 6).

As to the nature of the inclusions, or 'tingible Körper,' found in these cells, it might be assumed from their appearance and staining reaction that they were nuclei of degenerated small lymphocytes in this region, and such, indeed, is the case. The 'tingible Körper' of Flemming, then, are remnants of nuclei

of small lymphocytes which have been phagocytized by the acid-staining macrophages (fig. 5).

These macrophages, when first making their appearance in the nodules (ten to twelve days after birth), seem scattered about throughout the mass of lymphocytes, but as the nodule assumes a more definite shape and as the light central area becomes more prominent, it is seen that the macrophages tend to become confined to the central area. The so-called 'germinal center,' then, is an expression of two things: first, the lesser number of cells found here as compared to the peripheral portion of the nodule, and, secondly, the presence of acid-staining cells in greater abundance in this central portion (figs. 3, 4, 5).

As to the origin of these macrophages, no transitions between them and cells of the reticulum could be found (as by Weidenreich and Downey, and Hartmann). But, on the other hand, many transitional forms between cells of the lymphoid hemoblast type, with a basophilic cytoplasm and a large clear distinct nucleus, and the macrophages with an acid-staining cytoplasm and a nucleus somewhat indistinct because of its weak basophilic or almost acidophilic character. This change in nuclear and cytoplasmic staining reaction indicates, I believe, beginning degeneration of the cell. It is well known that many kinds of cells during a degenerative process acquire phagocytic powers. Maximow has proved, in study of tissue cultures of lymphatic nodes, that large lymphocytes (lymphoid hemoblasts) upon further differentiation acquire phagocytic powers. Therefore, it is considered that acid-staining macrophages in the light staining nodular center are the results of further differentiation and degeneration of lymphoid hemoblasts in that region.

Rather than being a place of special cell proliferation, then, the so-called germinal center seems to be a place of degenerative change. It is here where the degenerating small lymphocytes are found, the nuclear remnants of which are phagocytized by the macrophages, which themselves are apparently further differentiated, degenerating lymphoid hemoblasts.

The reasons that seem to the writer most plausible for this degenerative change are those of nutrition. The nodule arises

in a place of abundant blood supply, but as the nodule increases in size, due to differentiation and proliferation, the capillaries traversing the nodule become insufficient to nourish the larger amount of lymphatic tissue. The cells nearer the periphery, then, are better nourished than those more centrally located, because they receive nourishment also from blood vessels of the tunica propria and the submucosa. Therefore, degenerations are more numerous in the more centrally lying cells in a completely formed nodule.

The relationship between the system of lymph vessels and the nodules of lymphatic tissue in the intestinal tonsils is much the same as the relationship between the nodules of lymphatic and hemolymph nodes and the surrounding secondary sinuses. But in the lymphatic nodes the lymph in the sinuses bathes the lymphatic tissue directly, while there are no lymphatic sinuses in connection with the intestinal nodes. Injection of the lymphatic vessels (Berlin blue gelatin mass) shows, however, that there is a dense network of lymphatic capillaries just outside the connective-tissue sheath of the nodule. This is, of course, part of the submucosal plexus, which is, in turn, part of the system for transportation of the chyle (Heuer, Sabin). No lymphatics could be found penetrating the nodule in any case.

Among the things which must be considered in an interpretation of intestinal tonsillar tissue is the relationship existing between the lymphatic tissue and the overlying epithelium. Lymphatic nodules of the intestine do not remain restricted to definite areas, but spread in all directions from the point of formation. Thus the tunica propria in villi directly over lymphatic nodules become infiltrated with lymphocytes to such an extent as to form enlarged 'lymphatic villi.' After a certain stage in development (about fourteen days) has been reached, the lymphocytes begin to wander into the epithelium covering these lymphatic villi. It is, doubtless, this close association between the epithelium and the lymphatic tissue which led some of the earlier authors (as Retterer and von Davidoff) to the belief in an epithelial origin of these lymphocytes. Most authors, however, consider the relationship between the lymphocytes and the

epithelium to be secondarily acquired, due to the rapid growth of the lymphatic tissue. Jolly ('11), who noted the extraordinary infiltration of the epithelium over the intestinal tonsils, included them (i.e., the infiltrated epithelium) with the tonsils of the mouth and the thymus in one group, the 'lympho-epithelial organs,' in which he believes there exists a symbiotic relationship between the epithelial cells and the lymphocytes.

The lymphocytes, as stated above, first began to invade the epithelium to any extent about fourteen days after birth. Of course, from the very first appearance of lymphocytes in the tunica propria, some may be found in the epithelium but they do not invade it in great numbers till an age of two weeks or more has been reached. Until that time the basement membrane of the epithelium is quite complete, making a definite boundary between the epithelium and the underlying tissue, clearly indicating the impossibility of any of the elements of the lymphatic tissue taking origin from the cells of the epithelium. As more and more lymphocytes crowd into the epithelium the basement membrane becomes gradually less distinct, and the epithelium, at first was of a simple columnar type, gradually acquires the appearances of a heavily infiltrated, stratified epithelium, because of the displacement of the epithelial cells by the invading lymphocytes. Its thickness from cuticular border to basement membrane is greatly increased, but it really resembles an epithelium very little because of the huge number of contained lymphocytes. The invading cells, for unknown reasons, are all of the small lymphocyte variety. About each lymphocyte soon after it enters the epithelium is found a clear area, indicating some chemical activity by the lymphocytes upon the epithelial cells immediately surrounding them (fig. 12).

Not all of the lymphocytes found in the epithelium have wandered in from the underlying tissue, as there is evidence of proliferation of those which have already invaded it (fig. 12). Mitotic figures are occasionally seen in lymphocytes in this location. The spaces among the epithelial cells in which the lymphocytes rest are seen to often contain in later stages several small lymphocytes which have doubtless been derived by succes-



sive divisions of one which had invaded the epithelium. The lymphocytes in the intestinal tonsils, as opposed to those in the tonsils of the mouth and pharynx, rarely, if ever, wander through the epithelium into the intestinal lumen.

An interesting feature of the epithelium covering the lymphatic villi is the lack of goblet cells, which are so plentifully found elsewhere in the intestinal epithelium (Muthmann, Hartmann). In a study of the early developmental stages, it is found that goblet cells are numerous in the epithelial covering of all the villi. Glands (crypts of Lieberkühn) are not present as such until later (showing that goblet cells are not, necessarily at least, derived from the intestinal glands), but the epithelium between the bases of the villi is of a different character. In these regions no goblet cells were found. The places of nodular formation are in the tunica propria directly below places where there is an epithelium of this nature, and from these places the lymphatic villi arise usually, the epithelium being gradually forced upward between the ordinary villi as the lymphatic tissue increases in amount, the original character of the epithelium between the bases of the villi being maintained (i.e., without goblet cells). In a few instances, goblet cells were found in the epithelium of some of the lymphatic villi before its infiltration with lymphocytes occurred. The lack of goblet cells in the epithelium covering the lymphatic tissue is doubtless due to change in function of the epithelium, brought about by its relation to the lymphocytes (fig. 12).

The fundamental reasons for the development of lymphatic tissue, especially of the tonsillar lymphatic tissue, and the complete function performed by it are perplexing problems.

The only definite function which has been ascribed to lymphatic nodes is the production of lymphocytes. This surely is a function, but whether it is their only one is doubtful. Then the question arises as to whether tonsillar lymphatic tissue has other function than does the lymphatic tissue in other places. Some authors consider that, in the light of our present knowledge of tonsillar tissue, the only function that can be accurately ascribed to it is that similar to the function of lymphatic nodes; i.e.,

the production of lymphocytes (Brücke, '51; Hartmann, '14). In view of the fact that lymphatic tissue develops in places of degenerating body material, such as degenerating glands, rudimentary organs, etc., many authors thought the tissue as developing in these places assisted in absorbing and doing away with this degenerating material (Gulland, '91; Stöhr, etc.). Still others noted some relationship in the intestinal tonsils between the intestinal glands and the formation of lymphatic tissue, but were not sure of the nature of this relation (Flesch, '88; Klaatsch, '92).

It is well known that, in the presence of regressive structures, mesenchymal, or embryonic connective tissue shows a 'lymphocytopoietic' reaction. Accumulations of lymphocytes are encountered in glands of various kinds, as kidney, thyroid, salivary glands, minor digestive glands, etc., these accumulations being accompanied by degeneration and infiltration of certain of the glandular acini (Kingsbury, '15). Stöhr also calls attention to the fact that 'leucocytes' collect in places where organs are degenerating, as the pronephros of lower forms, gills of anura, thymus, processus vermiformis.

In the case of the rabbit, it is quite clear that the degeneration of any structures in the region of the intestinal tonsils does not initiate the formation of lymphatic tissue there. For no crypts of Lieberkühn are formed until lymphopoiesis is well under way. However, as the nodules of lymphatic tissue increase rapidly in size, they often include in their midst portions of intestinal glands, which are usually degenerating, this degeneration probably due to the infiltration of the lymphocytes, rather than the opposite. Any association observed here between the crypts of Lieberkühn and the lymphatic nodules is accidental, being due to the very rapid growth of the lymphatic tissue.

If the lymphatic tissue were to be interpreted as a lymphocytopoietic reaction of mesenchyme to regressive or degenerating structures, these structures must have been present at some time in the ancestral history, which in the animal, as existing now, do not appear at any time in its development.

It certainly seems as though the presence of a lymphatic plexus in the submucosa is insufficient to account for the extraordinary development of lymphatic tissue in Peyer's patch and the appendix, for this plexus is found throughout the small and large intestine and the stomach. It is noted that tonsillar tissue is greatly concentrated in and about the caecum. There may be some relationship existing between fecal matter in the caecum and appendix and the great development of lymphatic tissue here.

In discussing the functional possibilities of the intestinal tonsils, it may be recalled that the lymph vessels of the submucosal plexus form a fairly dense network of capillaries about the nodules. Lymphocytes are found quite abundantly in these vessels. The only source of these lymphocytes could be the lymphatic nodules of the tonsils, for there are no entering lymphatics which could carry them on. As the submucosal plexus is a part of the system for transportation of the chyle, it is evident, then, that the tonsillar tissue here serves, in part at least, in forming elements of the chyle.

#### GRANULOPOIESIS

It has already been pointed out that sometimes certain cells with granular content are abundantly present in the connective tissue of the wall of the intestine, especially in the region of the intestinal tonsils in and about the caecum. We are here able to study only the significance of those with acidophilic granules, as the basophilic granules of the mast cells have been dissolved out by the methods of tissue preparation used. Alcoholic fixation, which was found to preserve the basophilic granules, did not furnish material upon which their histogenesis could be determined.

The free eosinophile cells occur in varying abundance in the connective tissue of the submucosa and the tunica propria in the region of the intestinal tonsils, being found, however, most extensively in the deeper portions of the tunica propria, above and between the nodules of lymphatic tissue. They are often found in quite large numbers in the villi, closely associated with the intestinal epithelium, to a less extent in the connective-tissue sheath of the nodules and in the submucosa, and very rarely

in the midst of the nodules. They are usually found in quite intimate association with the blood vessels of the mucosa. In the appendix they are found in greater abundance than in the iliac tonsil of the same individual.

Modern hematologists are practically united in declaring for eosinophile leucocytes (granulocytes) an origin, either directly or indirectly, from cells of a lymphoid character. But, in respect to eosinophile cells of the intestinal mucosa, it must yet be decided whether they arise by local differentiation of lymphoid cells or have been carried in and dropped there by the blood stream.

Hartmann ('14) observed the large groups of granulocytes in the region of the intestinal tonsils, and he considered the majority of them to be true eosinophile leucocytes, with somewhat rodlike granules, and a polymorphous nucleus. He found, rarely, mononuclear forms with a light staining nucleus. He considered the eosinophiles found here as having been carried in by the blood stream.

Weill ('19), on the other hand, in studying the formation of the eosinophiles of the intestinal mucosa, favored the view that they were differentiated in situ from cells of a lymphoid character (lymphoid hemoblasts). He found transitional stages between the non-granular lymphocytes, through mononuclear granular forms, the 'eosinophilic myelocytes,' to a polymorphonuclear eosinophilic leucocyte. He declared that the young mononuclear forms, at least, could not have been carried in by the blood stream, for no cells of that type were found in the blood, all there being of the polynuclear or polymorphonuclear type.

Although hematologists are practically agreed as to the lymphoid origin of granulocytes, the question of the origin and significance of the acidophilic granules of the eosinophiles is still a much debated one. Several authors are of the opinion that eosinophiles are derived from neutrophils or other special cells by change in the granular character (van der Stricht, Gulland, Thayer, Arnold, Jolly, et al.). Others considered that neutrophils might transform into eosinophiles after taking up exoge-

nous material such as hemoglobin from extravasated red blood corpuscles (Klein) or products of degenerating muscle (Brown).

That the eosinophile granules of eosinophile leucocytes are phagocytized exogenous material of a hemoglobin nature is a belief held by many. This theory was followed by Schott, Gütig, Th. Lewis, Badertscher, and many others, but has been most vigorously defended by Weidenreich ('01, '08, '11, etc.). His conclusions were based on observations in hemolymph nodes and the spleen, and also on a series of experiments in which quantities of erythrocytes were injected into the peritoneal cavity of animals, where they undergo degeneration, breaking up into small particles which are phagocytized by large lymphocytes (lymphoid hemoblasts) of the peritoneal cavity and of the *tâches laiteuses* of the omentum (Weidenreich, '08; Schott, '09). In case there is no evidence of erythrocyte fragmentation, which Weidenreich admits is possible, he assumes the hemoglobin is released in solution, absorbed by the lymphocytes, and deposited in them in the form of granules. Badertscher ('13) found also that fragments of degenerating muscle may be phagocytized by lymphocytes, which then undergo a series of changes to become identical with blood eosinophiles. Some authors consider eosinophilic granules as related to hemoglobin in their nature, but they think are formed endogenously, and not introduced from without (Marwedel, '97; Pappenheim, '05).

Many of the recent investigators of this problem disagree with Weidenreich in his belief that the eosinophilic granules were exogenous material, but consider them true endogenous formations. Even here there is considerable difference of opinion as to the method of formation of the granules. Some (Danchakoff, Weill) thought the eosinophile granules were formed directly as such, appearing in the midst of the basophilic cytoplasm of lymphoid hemoblasts (according to Danchakoff, about the periphery of a slightly acidophilic centrosphere), the cytoplasm gradually losing its basophilic character as the granules increase in number. Maximow ('10) thought that the eosinophile leucocytes were not formed directly from lymphoid hemoblasts, but that first cells with 'pseudeosinophil' granules were formed

(‘Promyelozyten’ of Pappenheim), the granules of which later transformed into true eosinophile granules. Still others found the granules in these cells when first formed to be of a basophilic character, direct transformation and change later taking place, the staining reaction of the granules changing from basophilic to eosinophilic character, at the same time as other changes necessary to produce the adult eosinophile leucocytes are occurring (Downey, '15; Ringoen, '15).

The eosinophiles, occurring in the connective tissue of the tunica propria and submucosa of the digestive tract, in the region of the intestinal tonsils, occur in varying numbers, sometimes there being but a very few widely scattered, and again being found as large, concentrated masses, or granulopoietic foci. There is no definite time in development at which they appear in greatest abundance, this being apparently controlled by local conditions, varying according to the individual. They are, however, never present in great numbers until after the first week of postfetal life. Also, as stated before, they are almost invariably more abundant in the wall of the caecum and the appendix than in the iliac tonsil.

Their distribution in the connective tissue has already been spoken of. They are usually in greatest abundance in the tunica propria, especially around the bases of the typical, ordinary villi (figs. 10, 11, 16) (except isolated cases where large granulopoietic foci were found in the subnodular connective tissue).

A careful study of the eosinophile granulocytes as found in these places makes evident the fact that they are morphologically of two types, 1) the blood eosinophile and, 2) the connective-tissue eosinophile.

The first type, as the name given indicates, closely resembles the eosinophile leucocytes found in the blood stream. The cell is of a rounded form and contains a very polymorphous richly chromatic nucleus. The contained granules are rounded or slightly elongated (as those of the blood stream), and are packed quite closely together within the cytoplasm of the cell. With blood stains (as Hastings-Nochts) these granules stain a bright red color.

The other type of eosinophile found here is quite variable in shape according to its immediate surroundings, being sometimes rounded and again quite elongate, spindle-shaped, or irregular in contour. The nucleus is of an entirely different appearance than that of the first type, never being of an extreme polymorphous shape. It does, however, present variations in shape, sometimes appearing as one dense, round nuclear mass, and again appearing as two round masses, lying either side by side in the cell or widely separated at opposite sides of the cell. The cell body of these cells is very closely packed with particles, which are definitely rod-like and elongated. These rod-like particles stain an intense dark red when compound blood stains (Nochts Hastings, eosin-methylene blue) are used.

The first type, the blood eosinophile, is very evidently the same as Hartmann found in the neighborhood of the intestinal tonsils (of the rabbit). It is the first type to appear in the connective tissue at this place, the connective-tissue eosinophile not being found until between the first and second weeks of postfetal life. The blood type of eosinophile is never found in large numbers, except under pathological conditions (such as presence of parasites, etc.). It seems very evident that they, as Hartmann thought, are carried into the connective tissue and dropped there by the blood stream. No developmental forms are found to indicate a possible local formation of this type of cell.

On the other hand, there is evidence to indicate that the connective-tissue eosinophiles, found so abundantly in close relationship with the intestinal tonsils, are formed in situ at the expense of lymphoid hemoblasts also developing there (figs. 9, 10, 14, 16).

This process of development was found to be exceedingly difficult to follow because of the scarcity of developmental stages. This is accounted for by the great rapidity of the formation of the eosinophile granules after they have begun to differentiate out. Quite a few cells were found, however, the cytoplasm of which was only slightly basophilic, or colorless, which contained a few scattered, round eosinophilic granules (dark red). The nuclei of these cells were identical with those of lymphoid hemo-

blasts or slightly smaller (fig. 9). The mononuclear forms of rodded eosinophiles present the next step in nuclear differentiation, being similar to the 'hemoblast' type, but smaller and with more condensed chromatin material. By division of this nuclear material into two parts the binucleated rodded eosinophile leucocyte is produced. All transitional stages between mononuclear and binuclear forms can easily be found. First the round nucleus assumes an oval form, then an indentation appears in one side, and soon the two parts thus divided begin to pull apart, forming two lobes with a connecting strand. This connection seems gradually to disappear, so that there are apparently two distinct nuclear masses in the cell (figs. 10, 16).

In the production of granulocytes, then, the cytoplasm of involved lymphoid hemoblasts, the cells of origin, partially (as Maximow states) or entirely lose their basophilic character. After the basophilic character of the cytoplasm is lost, rounded eosinophile granules are formed endogenously in it, the nuclei at the same time decreasing in size. These granules increase rapidly in size and number, at the same time changing into a rod-like shape (in the rabbit). Soon the eosinophile particles fill all available space in the cytoplasm of the cells. Coincident with this is noted a continued decrease in the size of the nuclei with correspondingly increased density of the chromatin material. The change from the mononuclear to the binuclear form, as described above, apparently occurs after the cytoplasmic changes have been completed. This binucleated form is, as far as can be determined, the adult form of the connective-tissue eosinophile as occurring here.

Concerning the possibilities of homoplastic formation of eosinophiles, it certainly does not occur in the blood eosinophiles found in the connective tissue. No mitotic figures were seen in this type, as might be expected, for they are only present here in the adult condition. Mitotic figures in adult connective-tissue eosinophiles are never seen. They have been reported (by Dan-chakoff and Weill) in developing granulocytes or granuloblasts. The writer found no eosinophiles showing evidences of mitosis after granules had begun to be formed. This, then, as a source



of eosinophiles must be considered negligible; the main sources being by the heteroplastic differentiation of lymphocytes and from the blood stream.

The conditions causing or related to the phenomenon of granulopoiesis have not as yet been clearly brought out. The close association of granulopoietic foci with the blood vessels, which can usually be seen, indicates the probability that the blood in some way plays an active part in the formation of the eosinophiles. It is noted also that when parasites (as *Coccidia*) are present in any abundance that eosinophiles tend to become very numerous, especially in the tunica propria in the immediate vicinity of the parasites. The *Coccidia* may be found in groups near the bases of the villi, either imbedded in the tunica propria, just under the lining epithelium, in among the epithelial cells, or in the intestinal lumen, just outside the epithelium. The eosinophiles being formed locally or migrating from the blood vessels may invade the epithelium, or in case the parasites are found in the intestinal lumen, may break through the epithelium into the lumen where they gather about the parasites. The eosinophiles often invade the epithelium in such numbers that it is entirely broken up and loses its identity as such. The eosinophiles of both types always gather about and in the midst of the groups of *Coccidia* in an apparent effort to destroy them or combat their influence.

Other inflammatory or irritating conditions other than the presence of parasites may also be associated with extensive development of granulocytes. The fact that eosinophiles are more abundant in the appendix and caecum than in other parts of the intestinal tract may possibly be accounted for by their irritation caused by the presence of fecal matter in the intestinal lumen.

There is, therefore, surely some relationship existing between the presence of irritating substances, pathogenic organisms, etc., and the production of eosinophile cells. It is well known that eosinophiles from the blood collect in and about places of inflammation, but, in addition to that, inflammation or irritation of some kinds in the intestine seems to initiate granulopoiesis locally or the production of connective tissue eosinophiles. The more ex-

tensive the inflammation, the greater seems the production of eosinophiles.

The close association of blood vessels of the connective tissue with the groups of eosinophiles has already been mentioned. This is partially explained when it is recalled that some of these eosinophiles in the connective tissue are of the type found in the blood stream, which have wandered out through the capillary endothelium into the surrounding tissue. But there is an equally close association between the blood vessels and the granulopoietic foci of connective-tissue eosinophiles. Groups of granuloblasts (eosinophilic myelocytes) are always in the immediate neighborhood of some of the blood vessels of the mucosa. When the blood supply is increased as under inflammatory conditions of any nature, as caused by some irritant, such as presence of pathogenic organisms, the number of eosinophiles increases correspondingly. The greater the blood supply, the greater becomes the granulopoietic activity. It is very probable, therefore, that some agent in the blood stream (possibly in the plasma) plays an important rôle in the process of granulopoiesis. It may be suggested, therefore, that the underlying cause for excessive granulopoiesis is, then, the presence of some irritation bringing about essentially inflammatory conditions, the direct cause being some constituent of the blood stream (plasma?), the exact nature and action of which influence is unknown.

#### ERYTHROPOIESIS

The possibility of erythropoiesis occurring in the mesenchyme in various regions in the embryonic body has been noted by many hematologists.

Some of the earlier authors found in the subcutaneous connective tissue in some animals what they considered as intracellular development of red corpuscles. (Schafer, '74; Ranvier, '74; Le Boucq, '75.) This apparent intracellular development is now usually considered as an instance of the reverse process, atrophy of already formed vessels and breaking down of the contained erythrocytes by phagocytosis.

Typical erythropoiesis, however, has been observed by many authors in the mesenchyme or embryonic connective tissue in various places in the embryonic body. Saxer ('96) found it occurring in the mesenchyme in and about the musculature of the neck, in subcutaneous tissue, and various other places. Maximow found in rabbit embryos, it might be found in practically any place in the body mesenchyme, especially of the head region. Badertscher ('15), in study of pig embryos, found developing red blood cells in portions of the neck and upper thorax, as well as in the cortex and medulla, and interlobular septa of the thymus. Danchakoff also described erythropoiesis in the mesenchyme of the head in younger embryos, and in older stages also in the mesenchyme in other parts of the body.

Erythropoiesis, as occurring in these places, is mainly extravascular, although Danchakoff found it occurring both intra- and extravascularly. This, however, she does not consider as true, typical erythropoiesis for few or none of the thus formed erythrocytes get into the blood stream.

Aside from erythropoiesis occurring in the body mesenchyme, it is well known that it occurs in, or at least among, the mesenchymal or reticular cells in the embryonic liver and also in the embryonic spleen to some extent (in a few animals also in the adult spleen).

But the process of erythropoiesis, according to our present knowledge, is in postfetal life, limited to the red bone-marrow, with the exception of the first few days after birth, before the erythropoietic activity of the liver and of the spleen has ceased, and of the spleen, which may under pathological conditions (and in some animals, as the skunk, normally) reassume or continue its embryonic erythropoietic activity.

With this fact in mind, that, normally, after the second week of postfetal life, the sole seat of erythropoiesis known is the red bone-marrow (except in the spleen as stated), it is interesting to observe that, in studying sections from the appendix and the iliac tonsil of rabbits of an age varying from two to six weeks postpartum, there may be found in the connective tissue of the submucosa of these regions groups of cells in different stages of

the formation of erythrocytes. These erythropoietic foci are found mainly in subnodular and internodular connective tissue of the submucosa, just the opposite of the principal granulopoietic foci which are, in large part, found in the tunica propria above and between the lymphatic nodules. Erythropoietic foci are also of more frequent occurrence, and more extensive when present, in the region of Peyer's patch than in the appendix, again just the opposite of the granulopoietic activity.

Scattered erythroblast cells were also found in the subnodular and internodular connective tissue in stages both younger than two weeks and older than six weeks, but only during this period were erythropoietic foci of any extent found.

This close association between erythropoiesis and lymphoiesis is of exceptional interest because of the exceeding rarity of red-cell formation in connection with lymphatic tissue. One case of erythropoiesis occurring in lymphatic nodes was described by Pappenheim. This was found under pathological conditions, however, being associated with an acute case of hemorrhagic macrolymphocytic leukemia. Scattered erythroblasts have also been noted from time to time in hemolymph nodes. In the ileum and occasionally in the appendix of the rabbit one finds erythropoiesis occurring in no uncertain manner under apparently normal conditions in close relationship with the lymphatic tissue there present (figs. 7, 14, 15).

The developmental history of the three types of blood cells and possible relations between them has long been a perplexing question. The relation in origin between cells of the erythrocyte and granulocyte series has been particularly a matter of spirited controversy. On this question hematologists are divided in opinion into two groups.

The first group, believing in a monophyletic origin of blood cells, consider the erythrocytes and granulocytes are derived from a common stem cell, which, under certain environment, will develop in to the other type of blood cell.

The second group, on the other hand, believing in a polyphyletic origin of blood cells, consider that all types of cell of the blood series arise from stem cells of different character, which,

under any conditions of environment, will give rise to one type of cell only.

The monophyletic theory, first advanced by Saxer ('96) and Bryce, is, in some form, accepted by most of the more recent hematological workers.

Among those who most vigorously defend this monophyletic theory are Maximow, who made careful study of hematopoiesis as occurring in various places in the mammalian embryo, and Danchakoff who made investigations similar to those of Maximow in chick embryos, and also, more recently, studied experimentally produced erythro- and granulopoiesis in the spleen of the chick. Weidenreich, Pappenheim, in part, and many others also uphold this view.

With the histogenesis of erythroblastic tissue so clearly established by Saxer, Weidenreich, Pappenheim, and especially by Maximow and Danchakoff, it is unnecessary to discuss this in detail. A study of the regions in the connective tissue about the intestinal tonsils, where erythropoietic foci are forming, clearly indicates that the stem cells from which the cells of an erythroblastic nature develop, are identical, morphologically, with the lymphoid hemoblasts, or so-called germinal center cells of the lymphatic nodules, and, therefore, morphologically identical with the stem cell producing connective-tissue eosinophiles; i.e., a cell with a large, rounded or oval clear vesicular nucleus, with scanty chromatin, and one or more prominent nucleoli, and a variable amount of basophilic cytoplasm.

Subsequent changes of this type of cell to form an adult erythrocyte involve both the nucleus and cytoplasm. These changes of nucleus and cytoplasm are possibly not interrelated in any way, but they do occur simultaneously, and are brought about by conditions affecting the cell as a whole. Cytoplasmic changes are the gradual change from a basophilic to a brilliant acidophilic character, due to the laying down of hemoglobin in it. Nuclear changes are a gradual shrinkage in size, assumption of a pycnotic condition, and finally its extrusion from the cell. Many of the non-nucleated adult erythrocytes (erythroplastids) are found free in the connective-tissue spaces as well as developmental stages.

Presupposing the stem cells of the erythroblastic line of development to be elements of a lymphocytic nature (lymphoid hemoblasts) which are morphologically the same as the stem cells of the granuloblastic line of development, the question is raised as to what the factors are causing morphological similar cells to differentiate in such entirely different ways. Do the stem cells, as some polyphyletists say, possess only morphological similarity, with some inherent differences in them, by reason of which each must develop along a certain line? It seems more feasible to attempt to explain this difference in development by changes in environmental conditions, which would affect the metabolic activity of the cell.

In forms below the mammals a decided difference in environmental conditions is noted. Maximow has shown that erythrocytes develop intravascularly and granulocytes extravascularly in amphibia and selachians. It has been observed in studies of blood-cell development in birds and reptiles that almost invariably the erythropoietic foci are located intravascularly, the granulopoietic foci extravascularly (van der Stricht, Bizzozero, Danchakoff, and others). This relationship has been most carefully studied by Danchakoff. She did not, however, as van der Stricht, think the presence of an endothelial wall as sufficient evidence of the separate origin of these two types of cell.

If this were invariably true that erythropoiesis occurs intravascularly and granulopoiesis occurs extravascularly, that difference in environmental conditions would surely be sufficient to account for the different development of these cells; but, unfortunately, there seems to be an exception to this rule in the case of mammals, for it is a commonly accepted fact now among hematologists (except van der Stricht and a few others) that erythropoiesis occurs in large part extravascularly in mammals.

But if one finds similar cells under exactly the same environmental conditions developing into totally different types of cells, it strengthens the belief that there must be some inherent differences between these apparently similar stem cells. Stockard ('15) arrived at this conclusion after noting the extravascular formation of both types of blood cells in mammals. He found

also, in *Fundulus*, that erythropoiesis occurred only extravascularly, and that as soon as the erythropoietic tissue became included in a vessel, the process shifted to other regions of the embryo. The intravascular conditions he considered inadequate and inhibitory, rather than active factors for erythropoiesis.

As a partial solution of this apparent discrepancy in mammals might be advanced the results of Mollier ('13) in his study of erythropoiesis in the human embryonic liver. He found the endothelial walls of the sinusoidal vessels to be reticulated so that communication was made between the lumina of blood vessels and the immediately surrounding mesenchymal spaces in which the erythrocytes develop. Thus conditions in these spaces would be essentially intra- rather than extravascular.

Secondary extravascular erythropoiesis was also found to occur in birds (chick) by Danchakoff in the allantois following splenic grafts upon it. Some of the walls of allantoic blood vessels degenerated allowing the contents of the vessel, early stages of erythroblasts, to wander out into the mesenchyme forming extravascular erythropoietic foci. The cells thus liberated did not revert to lymphoid hemoblasts, but continued their development as begun intravascularly.

Using Maximow's experiments on the formation of bone-marrow in the kidney of the rabbit following ligation of the renal vessels, she applies this theory to mammals. Maximow found large groups of lymphoid hemoblasts collecting in the vessels due to the slower current caused by ligation which began erythropoietic differentiation intravascularly, and wandered out into the surrounding tissue at the normoblast stage. With this evidence at hand she thinks it probable that all extravascular erythropoiesis, whenever found, is only secondarily so, the process outside of the vessels being a homoplastic differentiation of specific cells (erythroblasts), irreversible in their development.

Erythropoiesis, as occurring in the submucosa in the region of Peyer's patch, in the rabbit, is definitely extravascular. Erythropoietic foci of very large proportions are often found at certain developmental stages of the tonsillar tissue, sometimes apparently filling most of the available space in the subnodular

and internodular connective tissue. Most of these foci do, it is true, contain cells in the later stage of erythroblastic development (normoblasts). But megaloblastic, extravascular foci may also be found. Cells in the same group are often at various stages of erythroblastic development (figs. 8, 14, 15).

If this extravascular erythropoiesis were secondarily so, one would expect to find at some developmental stage intravascular megaloblastic foci, or at least intravascular groups of lymphoid hemoblasts, but at no time were such found. The entire process of erythropoiesis as it occurs here is apparently extravascular.

No evidences were found to indicate that the walls of the submucosal blood vessels were reticulated so as to make the mesenchymal spaces connected with the lumina of the blood vessels. This is true extravascular erythropoiesis, and not secondarily so, nor occurring under conditions of intravascularity (as Mollier described).

Though this erythropoiesis is extravascular, erythropoietic foci are always seen to be in very close relationship with the blood vessels of the submucosa. There is little doubt but that some action of the plasma or some element contained in it upon lymphoid hemoblasts locally developed incites them to further differentiation along the erythroblastic line of development. This seems to be very similar to the granulopoietic relations before described, but it is found that this relationship between erythropoietic foci and the blood vessels is much closer than between the granulopoietic foci and the blood vessels. There certainly is, however, very great similarity between granulopoiesis and erythropoiesis, and only a slight difference in environmental conditions must exist.

Both granulopoiesis and erythropoiesis are found to occur in largest extent when the blood vessels are gorged with erythrocytes (i.e., when the current in the blood stream is slow). If one considers the various places in the body of the embryo and adult where these two phenomena are known to occur, as in the liver, spleen, bone-marrow, etc., these are found to be invariable places where the blood current is slow, the slowing of the current usually being due to the sinusoidal nature of the vessels. This slowing



of the current, together with the thinness of the vascular walls, usually only a lining endothelium, affords excellent opportunity for the transudation of substances from the blood stream to the outlying near-by lymphoid hemoblasts.

It seems very probable, though, that the complete explanations for these erythropoietic tendencies are deeply seated.

Elements are present in the blood stream in certain definite proportions. A disturbance of this balance between elements in any way, as by change of conditions, destruction of some of the elements, etc., causes initiation of erythropoiesis to restore that balance. The submucosal connective tissue in these regions affords an excellent place for this to occur, for here stem cells (lymphoid hemoblasts) are being produced, the current of the blood stream is slow, the blood vessels are numerous, and the vascular walls, to a great extent, quite thin. Then, too, the connective tissue is here performing no very active function, and ample space is provided between the bases of the nodules and the muscle coats (especially in Peyer's patch) for the development of erythropoietic foci.

#### DISCUSSION OF THE RELATIONS CONCERNED WITH THE FORMATION OF THE BLOOD CELLULAR ELEMENTS

The formation and development of the three different types of blood cells, then, and relations causing or connected with the appearance of each type evidently are very closely associated.

Lymphopoiesis, granulopoiesis, and erythropoiesis are all closely associated with the vascular system. Lymphopoiesis, of course, must be the first process to occur, for it is from stem cells of a lymphoid nature that the cells of the granuloblastic and erythroblastic series are developed. This lymphopoietic process is, apparently, in some way, initiated by the lymphatic vessels, free lymphoid cells (lymphoid hemoblasts and small lymphocytes) forming by differentiation of mesenchymal cells. The subsequent fate of these cells, thus formed, is dependent upon the blood supply and the closeness of their relation with the blood vessels. If there seems to be no particular association with the blood vascular system, these free cells continue to

develop in a somewhat random fashion, and the result is a diffuse lymphatic tissue. If there is a very good blood supply giving excellent nutritive conditions for growth and proliferation, there is a rapid increase in the number of free cells in regions immediately about the blood vessels, so that dense, nodular lymphatic tissue is formed.

After cells of the lymphocyte series are formed (lymphoid hemoblasts and small lymphocytes), some of them may become more intimately associated with some of the large thin-walled blood vessels of the mucosa or submucosa, in which the current of the blood stream is quite slow, and differentiate further into either granular leucocytes (eosinophilic) or erythrocytes, according to the closeness of this relationship, and the degree to which the conditions of the vascular wall and the current of the blood stream make the transudation of the necessary materials from the blood stream possible. This is also governed by the distance of the hemoblasts from the source of materials, the blood vessels, those farthest from the vessels having granuloblastic tendencies and those nearer to them erythroblastic tendencies. No small lymphocytes, however, develop directly into either granuloblasts or erythroblasts, always first by growth, developing into cells of the lymphoid hemoblast type.

These factors influencing further development and differentiation of lymphoid hemoblasts may account for the greater concentration of granuloblastic cells in the tunica propria and the greater concentration of erythroblastic cells in the submucosa in the region of the intestinal tonsils. For the submucosal vessels are larger and more sinusoidal and comparatively much thinner walled than those in the tunica propria. The slower current, due to the more sinusoidal character of the vessels, coupled with the comparative thinness of the walls, affords better opportunities for transudation of materials from the blood stream, so that conditions are better for erythropoiesis in the submucosa. Similar conditions, to a lesser degree, exist in the tunica propria, so that here the process most usually taking place is granulopoiesis.

## SUMMARY

1. The first free cells appearing in the process of the formation of lymphatic tissue in the intestinal tonsils are mainly small lymphocytes, which arise by differentiation of fixed mesenchymal cells. A few of the large mesenchymal cells, however, transform directly into large lymphoid hemoblasts. These two types are apparently different growth stages of the same cells, the small lymphocytes by growth and slight differentiation becoming lymphoid hemoblasts, and the latter by repeated divisions becoming reduced to small lymphocytes. Further indication of their close relation is the fact that either type may arise by transformation of mesenchymal cells, according to the size of the transforming cell.

2. The so-called 'germinal center' is not really a center of proliferation of lymphocytes as that name would indicate. The lighter appearance of the central portion is partially due to the looser arrangement of cells there than at the periphery where the cells pile up as a result of their outward spread being limited by the surrounding connective tissue. The other factor producing this lighter appearance is the greater number of large acid-staining cells in this position.

3. The acidophilic cells are found to arise by further differentiation and degeneration of lymphoid hemoblasts. Experimentation proves that they possess phagocytic powers. Inclusions found in their cytoplasm ('tingible Körper' of Flemming) are considered as nuclear remnants of degenerating small lymphocytes which have phagocytized by the degenerating lymphoid hemoblasts, the acid-staining macrophages. Indications are, then, that this lighter central area is essentially a degenerative center, brought about possibly by poorer nutritive conditions in the center of the nodule.

4. The intestinal epithelium overlying the lymphatic tissue begins to be infiltrated with lymphocytes at an age of about fourteen days. The number of invading cells rapidly increases both by proliferation of those already in the epithelium and by continued invasion from the lymphatic tissue. The nature of

the relation between the epithelial cells and the lymphocytes is not definitely known. The lymphocytes rarely, if ever, pass through the epithelium into the intestinal lumen.

5. The granulocytes (eosinophilic) as found in the region of the intestinal tonsils are of two types, 1) blood eosinophiles, wandering into the connective tissue from the blood stream, and, 2) connective-tissue eosinophiles, being derived in situ by differentiation of lymphoid hemoblasts.

The granules of the connective-tissue eosinophile are endogenous formations. They appear first as round bodies, later changing to a rod-like form. The formation of granules is preceded slightly by loss in the basophilic character of the cytoplasm. No change in staining reaction of the granules during the course of their development was noted. Nuclear changes follow those of the cytoplasm. The nucleus first becomes smaller and finally divides into two, the binucleated condition being, apparently, the adult condition.

6. Granulopoiesis is most active in animals infected with parasites, as *Coccidia*. It also occurs most abundantly in and about the caecum, where there may be assumed to be a constant irritation due to the presence of fecal matter. It may be suggested, then, that inflammatory conditions may be associated with a granulopoietic reaction.

7. Extravascular erythropoietic foci occur in abundance at certain stages in the development of the lymphatic tissue. These are extravascular throughout their entire development, at no time being within the blood vessels. No groups of lymphoid hemoblasts or megaloblasts were found intravascularly at any time of development. These erythroblastic cells also arise by differentiation of lymphoid hemoblasts.

8. It is evident that conditions associated with the development of the three types of blood cells are closely interrelated, all being doubtless in some manner associated with the vascular supply. The initiation of lymphopoiesis is apparently brought about by some influence of the abundant lymph vessels or the lymph upon the mesenchyme. The formation of nodular lymphatic tissue may possibly be controlled by the nutritive conditions furnished by the blood supply. The further differentiation

of lymphoid hemoblasts into cells of the granuloblastic or erythroblastic line of development may be conceived as dependent upon the closeness of association between the blood vessels and the lymphoid hemoblasts, the slowness of the current of the blood stream, and upon the thinness of the vascular walls. If the association between the lymphoid hemoblasts and the blood vessels is very close and the conditions necessary for the transudation of materials from the blood stream (plasma) are very good, erythropoiesis occurs. If the association is not so close or the transudation conditions present to a lesser degree, granulopoiesis results.

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## PLATE 1

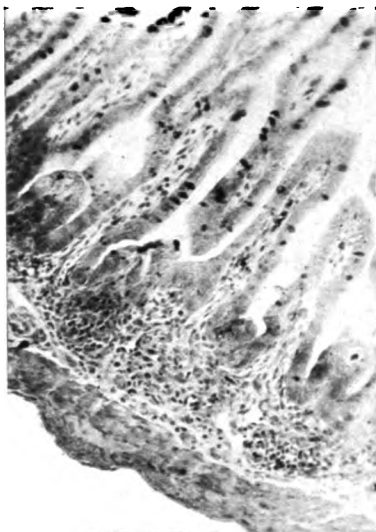
### EXPLANATION OF FIGURES

1 Rabbit, age five days. To show the position of the first masses of free cells appearing in the region of Peyer's patch. Photo  $\times 180$ .

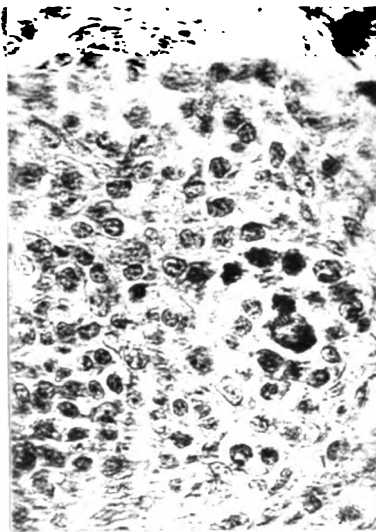
2 Rabbit, age nine days. To show the consistency of these masses. With the exception of one lymphoid hemoblast, all shown are of the small lymphocyte type. Photo  $\times 750$ .

3 Rabbit, age thirty-two days. A portion from the center of an intestinal lymphatic nodule of Peyer's patch. Note how loosely packed the cells are here as compared with the peripheral portion of the nodule (fig. 4), also the scarcity of mitotic figures.

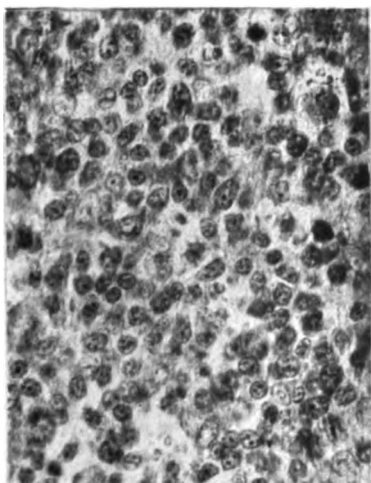
4 The same as figure 3: From the peripheral portion of the nodule. The lymphocytes of both types are very densely packed here. Mitotic figures are numerous.



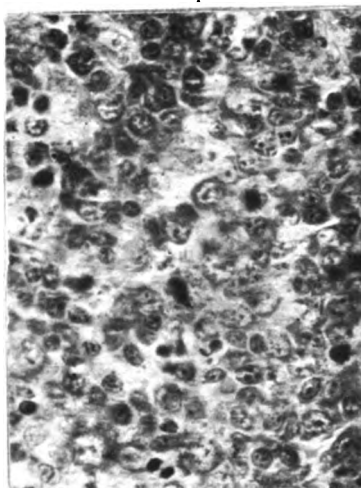
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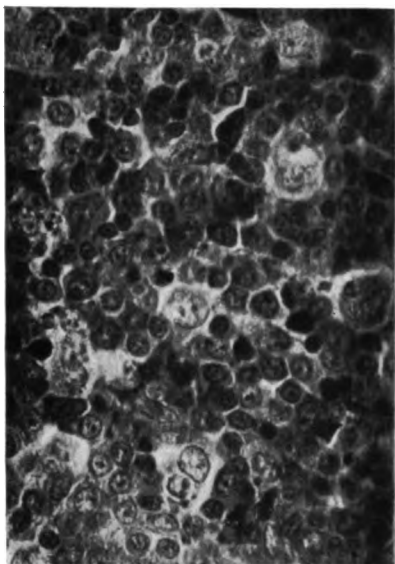
## PLATE 2

### EXPLANATION OF FIGURES

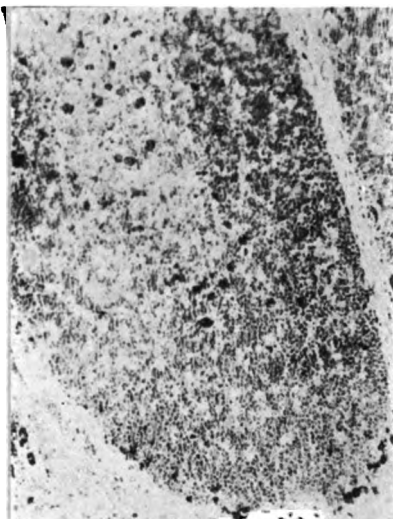
5 Rabbit, age forty-six days. A portion from the center of a nodule of Peyer's patch, showing the abundance of acidophilic macrophages (degenerating lymphoid hemoblasts), some with nuclear remnants of small lymphocytes as inclusions. Other degenerating small lymphocytes are also found. Photo  $\times 750$ .

6 Rabbit, adult. To show the appearance of a nodule of Peyer's patch, following injection of a 5 per cent trypan blue solution into the intestinal wall. The acidophilic macrophages which are found chiefly in the central portion have phagocytized large quantities of the dye. Photo  $\times 180$ .

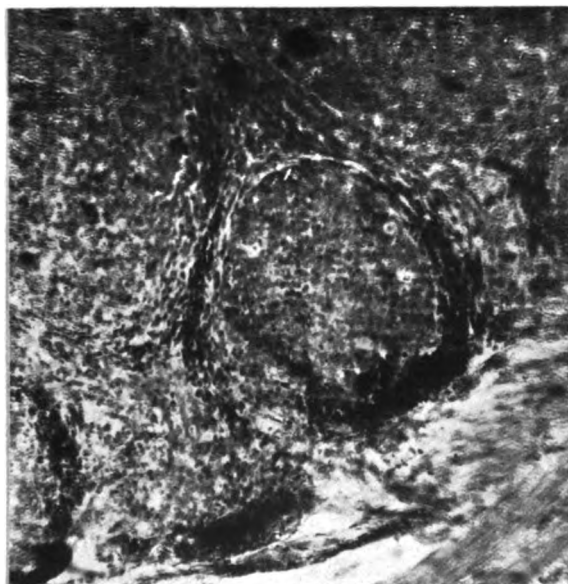
7 Rabbit, age twenty-eight days. Showing very extensive erythropoietic foci in the sub- and internodular connective tissue in the region of Peyer's patch. These foci appear as dark masses of cells. Photo  $\times 180$ .



5



6



7

### PLATE 3

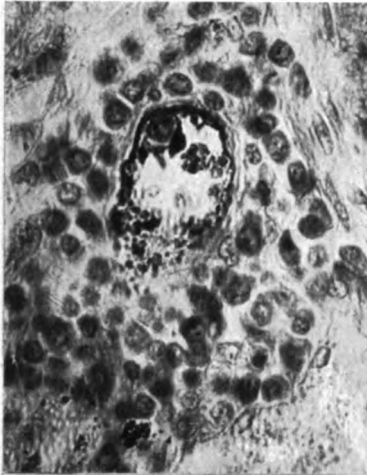
#### EXPLANATION OF FIGURES

8 Rabbit, age forty-four days. Erythroblastic cells in close association with a small blood vessel in the region of Peyer's patch. Photo  $\times 750$ .

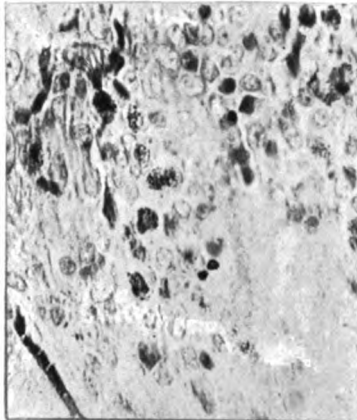
9 Rabbit, age forty-four days. Mononuclear eosinophiles in the tunica propria of the caecum near its junction with the ileum. Early developmental stages in their formation are seen. Photo  $\times 750$ .

10 Rabbit, age twenty-one days. Mononuclear and binuclear eosinophiles in the tunica propria, directly underneath the epithelium, in the region of the appendix. In some cells the eosinophilic granules are so densely massed as to make the cytoplasm appear in the photograph as almost homogeneous. Photo  $\times 750$ .

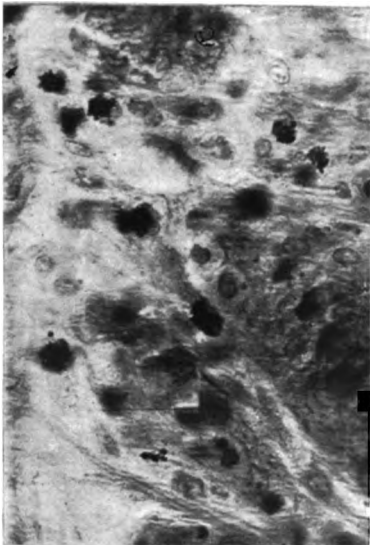
11 Rabbit, age twenty-one days. Eosinophiles in the tunica propria and in among the epithelial cells in the region of the appendix. Photo  $\times 750$ .



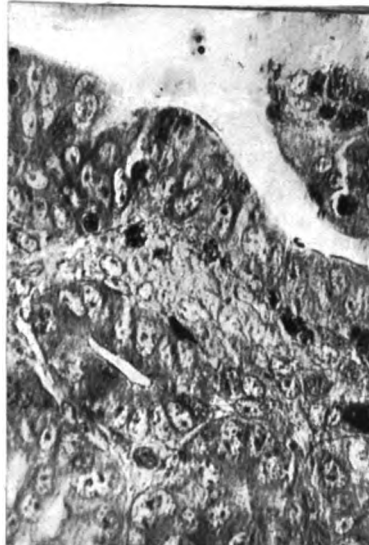
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## PLATE 4

### EXPLANATION OF FIGURES

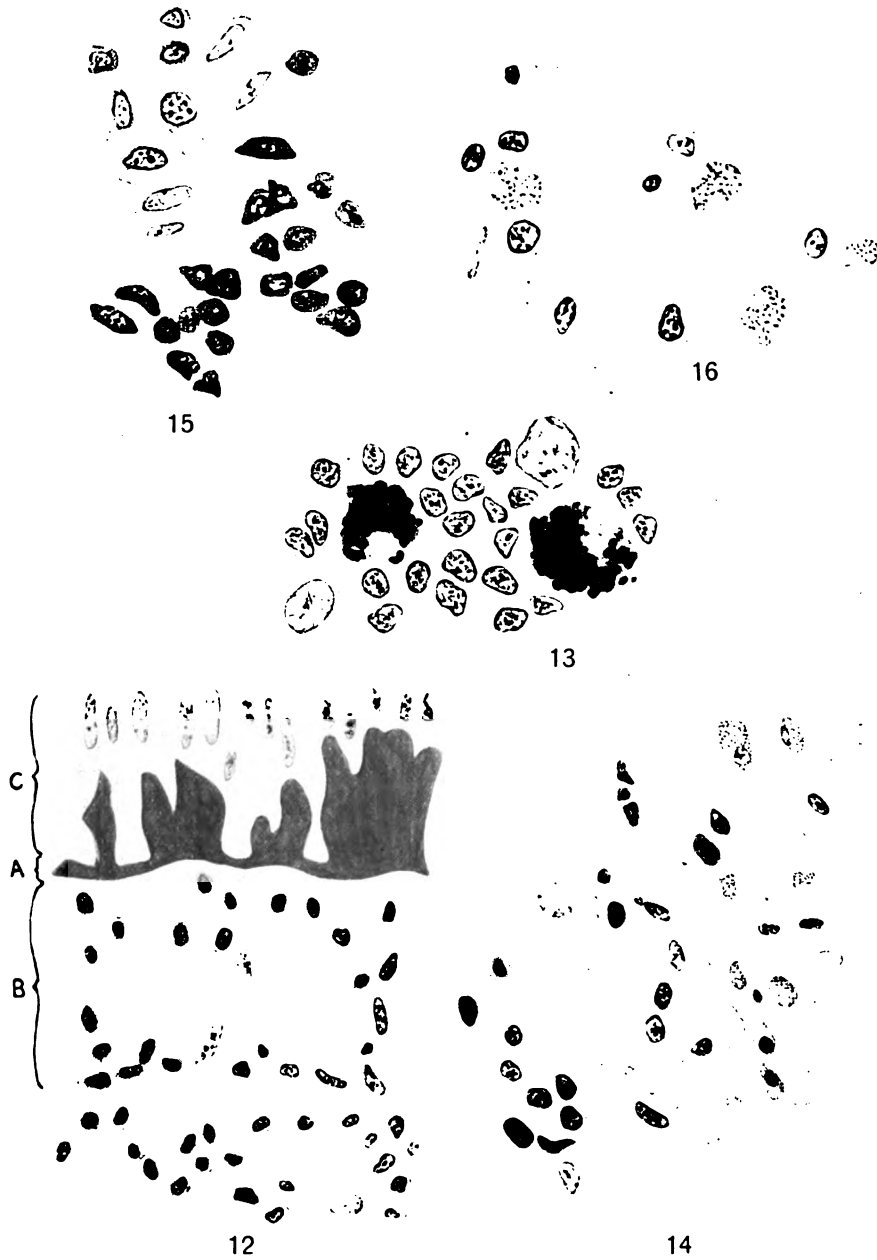
12 Rabbit, adult. Showing the heavy infiltration of small lymphocytes in a portion of the epithelium covering a lymphatic nodule of Peyer's patch (*B*). These invading cells occupy spaces in the epithelium, with a varying number of cells in each space. The nuclei of four epithelial cells are indicated. The upper part of the drawing shows a portion of the epithelium of a neighboring villus (*C*); with numerous goblet cells pouring their secretion out upon the free surface (*A*). Note the absence of goblet cells in the epithelium covering the nodule (*B*). Wright's stain.  $\times 1400$ .

13 Rabbit, adult. Cells from the center of a lymphatic nodule of Peyer's patch following an injection of a 5 per cent aqueous solution of trypan blue. Two acidophilic macrophages which have phagocytized particles of the dye are shown. Two normal lymphoid hemoblasts are also indicated. Stained with safranin.  $\times 1500$ .

14 Rabbit, seventeen days. Erythroblastic and granuloblastic cells in the subnodular connective tissue in the region of Peyer's patch. The nuclei of the erythroblasts are pyknotic. Several erythrocytes which have lost their nuclei are seen. Two lymph vessels and one arteriole are present. The erythroblastic cells are in close association with them, while the granuloblastic cells are more distant from them. Stained with eosin-methylene blue.  $\times 1400$ .

15 Rabbit, seventeen days. A portion of a large erythropoietic focus in the subnodular connective tissue in the region of Peyer's patch. Several lymphoid hemoblasts from the adjacent nodule are indicated in the drawing. Stained with eosin-methylene blue.  $\times 1400$ .

16 Rabbit, forty-four days. Mononuclear (granuloblasts) and binuclear (granulocytes) eosinophiles in the tunica propria of the intestine in the region of Peyer's patch. Note the rod-like appearance of the granules. Several lymphoid hemoblasts are also present. Hastings-Nochts stain.  $\times 1400$ .





Resumen por el autor R. R. Humphrey.

### Las células intersticiales del testículo de los Urodelos.

El testículo de los Urodelos constituye un material particularmente favorable para el estudio de las correlaciones entre el desarrollo de las células intersticiales y las fases del ciclo espermatogénico: en él los cambios progresivos y regresivos de la espermatogénesis están separados en un grado raro o nunca aproximado en el testículo de los vertebrados superiores. El desarrollo de las células intersticiales en los urodelos adultos está asociado con cambios regresivos en el testículo al final del ciclo espermatogénico. Durante la espermatogénesis los lóbulos están rodeados solamente por células estromales aplanadas o fusiformes. Cuando son expulsados los espermatozoides estas células aumentan en número y volumen, acumulando lípidos en su interior y transformándose en células intersticiales típicas, persistiendo como tales células durante tiempo variable en diferentes urodelos, asociadas con las porciones vacías y degeneradas de los lóbulos. Su degeneración final o reversión al tipo de células del estroma tiende a seguir más o menos estrechamente la desaparición de tales lóbulos en vías de degeneración. Es evidente que las células intersticiales no pueden desempeñar función trófica alguna durante las fases progresivas del ciclo sexual siguiente puesto que su degeneración o reversión puede ocurrir muchos meses antes de comenzar la espermatogénesis (es decir el desarrollo de los espermatoцитos primarios). Tampoco establece este estudio relación causal alguna entre su desarrollo y la aparición de los caracteres sexuales secundarios o instintos de copulación. Su desarrollo alrededor de los lóbulos en vías de degeneración después de la expulsión de los espermatozoides es el rasgo más saliente de su historia en todos los urodelos estudiados.

Translation by José F. Nonidez  
Cornell Medical College, New York

# THE INTERSTITIAL CELLS OF THE URODELE TESTIS<sup>1</sup>

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SEVEN TEXT FIGURES AND FOUR PLATES (FIGURES EIGHT TO THIRTY-NINE)

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## INTRODUCTION

The frequent occurrence of the term 'interstitial gland of the testis' in the extensive current literature on internal secretions indicates that the secretory function of the interstitial cells of Leydig has become widely accepted as a demonstrated fact.

<sup>1</sup> Expenses incurred in the course of this investigation have been partially met from the Mrs. Dean Sage Research Fund; this assistance is hereby gratefully acknowledged.

The assumption that the 'secretion' of this 'gland' is responsible for the development of the secondary sex characters or sexual instincts, or both, follows. The mass of evidence supporting such an assumption is, however, entirely indirect, and is frequently conflicting when several forms are considered. The difficulties lying in the path of the worker will be suggested by the following comments upon several lines of investigation and experiment.

The effects of castration are too well known to need description, but the operation removes all testicular structures; the injection of testicular extracts, though demonstrating clearly the presence of elements capable of influencing sex characters and instincts, does not limit the source of this element to the interstitial cells; vasectomy as practiced on laboratory animals and man (Myers, '15) does not result in complete exclusion of other possible sources of the secretion which causes retention of the sex characters and instincts in animals so treated, since Sertoli cells as well as interstitial cells are found to survive. The same may be said of x-ray treatments, which, like vasectomy, destroy the germinal epithelium, but do not eliminate the cells of Sertoli.<sup>2</sup> But as histologic studies, in such cases, show apparent increases in number or size of the interstitial cells, and partial or even complete<sup>3</sup> degeneration of the Sertoli cells, it has been concluded that the former rather than the latter must produce the secretion, if any be produced.

So, too, do the studies of cryptorchid testes and the organs of hermaphrodites fail to give clearly convincing evidence. Hanes ('11) believed that in cryptorchid pigs the evidence from the sex characters of animals and histological studies of the testes warrants the belief in the secretory function of the interstitial cells; Whitehead ('08), from studies on a cryptorchid stallion, inclined to the same opinion, and, further, considered that the Sertoli cells had been ruled out as a possible source of secretion because of their degenerate condition in the animal studied.

<sup>2</sup> Biedl, page 396, lists numerous workers in this field and summarizes their findings.

<sup>3</sup> Tournade ('03-'04) has shown that degenerative destruction of the Sertoli cells ultimately results from ligature of the vas deferens in rats.

Bell ('16), on the other hand, is emphatic in the statement of his belief that the interstitial cells cannot be responsible for secondary sex characters, as he finds these cells developed "in the testes of tubular partial hermaphrodites with feminine secondary sex characters . . . . to a degree that is rarely seen in the undescended testis and never in the normal testis;" Boring and Pearl ('18) conclude from their study of hermaphrodite fowls that "interstitial cells are clearly shown to have nothing to do with any of the secondary sex characters."

Considerable attention has been given to the embryonic development of these cells, as well as to the periods at which, in the life of the individual, their maximal development is attained. Their prominence in human embryos and their relatively smaller bulk in the eight-year-old boy were noted by Hofmeister ('72). Kasai ('08), in a very complete series of 130 human testes, found that the increase in the interstitial cells which occurs at puberty is followed by an apparent decrease; the reduced number peculiar to the period of sexual activity is then followed by an increased number during old age. Such a condition is difficult of explanation if one assumes the responsibility of these cells for the development of sex characters or instincts; neither is the complete disappearance of these cells in adult male fowls, as reported by Boring and Pearl ('17), and confirmed by Nonidez ('20), in accord with such an assumption.

#### CYCLIC CHANGES

##### *Historical*

The cyclic changes of the cells in animals which mate only at widely separated periods have recently attracted the attention of investigators. Studies have been undertaken to determine in what relation their maximal development stands to the periods of the sexual or spermatogenetic cycle; a constant relation, if established, might serve as evidence of the production of an internal secretion or give a clue to its possible influences. These investigations are here briefly reviewed in order that the varying results thereof may be more clearly brought before the reader.

Hanseman ('95), the earliest worker in this field, reported few or no interstitial cells present in the marmot during hibernation, when spermatogenesis is not active. In the spring, after several weeks of active life and the resumption of spermatogenesis, they are greatly increased in numbers. Ganfini ('03), on the other hand, declared the interstitial cells of the marmot during hibernation to be fully as numerous as during the animal's active period, but to be smaller and of different structure and staining reactions. He did not consider these differences as in any way correlated with spermatogenesis, but thought them due to changed conditions of metabolism, such as might affect any other tissue during hibernation.

Regaud ('04), Lecaillon ('09), and Tandler and Grosz ('11) worked on the testis of the mole (*Talpa europea*). They found that spermatogenesis goes on during the autumn and winter months, during which time the interstitial cells are at their minimal size and number. After the spermatozoa leave the testis in March, the tubuli contorti become reduced in size, while the interstitial cells increase greatly in size and number, their vacuolated cytoplasm at this time indicating the presence of lipoids. With the increase in spermatogenesis in the early autumn, a reduction in the size and number of interstitial cells occurs.

Marshall ('11), examining the testes of hedgehogs (*Erinaceus europaeus*), found no spermatogenesis during winter, but a rapid enlargement of the testis in the spring preceding the rutting period, this enlargement being due to growth of the interstitial tissue even more than to increase in size of the tubules. Regressive changes begin in October, and the cells soon become so reduced in quantity as to bring the tubules into contact.

Rasmussen ('17) found that in the woodchuck (*Marmota monax*) spermatogenesis is proceeding very slowly during the period of hibernation. The interstitial cells are then smallest in size and probably reduced in number; following upon the renewal of activity in the tubuli contorti, in the spring, the interstitial cells undergo a period of rapid development, not, however, attaining their maximum until several weeks after free sperma-

tozoa have appeared, and then persisting in this greatly developed condition fully two months after the spermatogenetic cycle has ended.

It would appear from the above-cited results that considerable variation occurs in the relations between interstitial cell growth and spermatogenesis in the testes of mammals: the two may appear directly correlated, as in the marmot and hedgehog; regressive changes in the germinal tissue may be accompanied by the maximal development of the interstitial tissue, as in the mole; or interstitial cell development may follow rather closely after the period of greatest activity in the tubuli contorti, as in the woodchuck. Regarding this variation, Rasmussen says: "This seems to be the most striking correlation, namely, that the interstitial cells follow with renewed growth somewhat behind the spermatogenetic cycle." He considers it possible, in this connection, that in the mole they "are only somewhat more than usually behind."

The same lack of uniformity is found in any of the higher vertebrate classes of which enough members have been studied to render comparisons possible.

Very little has been done among birds. The only complete study of a member of this class was made by Stieve ('19), who worked on the testis of the corvus, an European bird of the raven type. He found that the interstitial tissue appeared more prominent in the testis in the months following the breeding period, when the tubules had become relatively shrunken; but he concluded that there was no actual increase in the bulk of the interstitial cells at this period.

No observations on the seasonal changes in reptiles have been encountered save the statement of Mazetti ('11) that interstitial cells were abundant in the testes of hibernating snakes.

Several investigators have published accounts of the cyclic changes in the testes of anurans. Friedmann ('98) found the development of interstitial cells running parallel with the progress of spermatogenesis in the frogs (*Rana fusca*, *Rana viridis*, *Hyla arborea*) and the toad (*Bufo vulgaris*). When spermatogenesis has practically ceased, the interstitial cells tend to become re-

duced to their minimal size and number. When, on the other hand, spermatogenesis is at its height and free spermatozoa most numerous, the interstitial cells are of maximal size. Mazetti ('11) confirms these observations of Friedmann with regard to the frogs *Rana fusca* and *Rana viridis*.

Champy ('08, '13) found in another species of frog (*Rana esculenta*) that the maximal development of the interstitial cell occurs when spermatogenesis is at its lowest, and vice versa. This condition would appear to differ greatly from that reported by Friedmann and Mazetti in the three species of frogs studied by them. Champy found in the toad (*Bufo vulgaris*) a condition similar to that in *Rana esculenta* and quite the opposite, of that described by Friedmann. He also states that in *Rana temporaria* the interstitial cells develop only after mating and disappear entirely during the progress of spermatogenesis.

#### *Significance of variation in correlations*

There is, then, in the higher vertebrates, a decided lack of uniformity in the relations between interstitial cell growth and the progress of spermatogenesis, the variation existing even between closely related members of the same class. The explanation of this condition, doubtless, is to be sought in the complex relations within the tubules of the testis. In mammals, birds, reptiles, and anurans, the tubuli contorti are lined at all times by a germinal epithelium, from which the germ cells are proliferated. In animals which breed at any time of the year spermatozoa are being matured constantly and shed into the lumen of the tubule. Around the periphery of the tubule, between the lumen and the basal layer of Sertoli cells, spermatogonia persist through all the changes involved in the maturing of the spermatozoa, and before the ripe spermatozoa at any one point have been shed into the lumen, these will already have differentiated, so that spermatocytes will be developing between basal spermatogonia and the lumen. In closely adjacent parts of the tubule will be found other stages, so that spermatogonia, spermatocytes, spermatids, and free spermatozoa, as well as the Sertoli cells,

will all be present in a limited area of the tubule. Progressive and regressive changes, therefore, must go on side by side, rendering impossible any accurate analysis of conditions which may affect interstitial cell development.

Animals which have but one rutting period yearly offer more favorable material for study, since in these the various stages of spermatogenesis are more widely distributed as to time. But even in testes of such animals (when we deal with mammals, birds, reptiles, or anura) there is no absolute separation of the progressive from the regressive changes such as would enable one definitely to know with which the interstitial cell growth is correlated. Degenerative changes, for example, occur when the transforming spermatids cast off a part of their cytoplasm, but such changes cannot affect adjacent testicular structures which may not at the same time, be subject to influences due to the progressive changes in the spermatogonia, or be profoundly affected, also, by the varying processes transpiring in the omnipresent Sertoli cells.

If, then, the interstitial cell status at any period is the expression of conditions within the tubules, it must be the expression of several influences acting synchronously rather than of one influence acting without interference. These combinations of influences may be thought of as acting as do the combinations of forces of the physicist: opposing forces tend to neutralize each other, and variations in the relative strengths of forces to change appreciably the direction or extent of the resulting movement. That different animals should differ in details of metabolism is inevitable. A degenerative change, for example, may be slightly delayed in one species and accelerated in another; such a change, then, considered as a force capable of affecting interstitial cell development, is differently applied in the two animals and may lead to a difference in their interstitial cell development—a difference, it is true, subject both to increase and diminution by the action of other influences in the combinations. It is, I think, to some such explanation as this that we must turn if we are in any way to harmonize the apparently varied results of previous investigations of cyclic changes.



The fact remains that such variations in cyclic changes do exist, as well as other differences, more or less difficult of explanation. A survey of the situation leaves one with these questions:

1. Are the variations noted due to fundamental differences in the cells themselves in different animals, or are they due to the varying 'balance' of many conditions, each one of which is more or less obscured in complex testes?

2. Do the testes of any lower vertebrate forms not yet investigated possess a structure in which the processes are more separated, so that the correlations of the interstitial cells may be more easily interpreted, and, if so, to what is their maximal condition in such forms related?

It is with the questions last stated that this investigation is chiefly concerned, for a satisfactory answer thereto may be expected to throw some light upon the difficulties encountered in testes of the more complex type.

#### PRESENT INVESTIGATION

Kingsbury ('14), at the conclusion of a paper on the interstitial cells of the mammalian ovary, discussed briefly the corresponding cells of the testis. He called attention to the greater difficulty involved in the male than in the female organ in analyzing the conditions underlying their appearance; he further insisted upon the need for work on testes of lower forms in which the progressive and regressive changes are more widely separated in time and place, as they are in the ovary of the female. The urodele amphibian possesses such a testis. In it, as will be made apparent subsequently, the growth processes and degenerative changes are isolated to an extent not found in any higher vertebrate, no matter how widely separated its rutting periods may be.

To Dr. Kingsbury the writer is indebted for suggesting this problem and for the generous contribution of the urodele material he had collected. His helpful suggestions and criticisms during

the course of the investigation, as well as those of Prof. S. H. Gage, are gratefully acknowledged.<sup>4</sup>

### *Material and technique*

The material upon which this investigation is chiefly based consists of the testes of fifty-two specimens of *Necturus maculosus*, of which forty-nine animals were sexually mature. The three immature animals were each of about 21 cm. body length; the sexually mature animals ranged in length from 22 to 37 cm. Twenty-five of these animals, including the three sexually immature specimens, were taken from Cayuga Lake; the remaining twenty-seven were secured from Lake Erie at Venice, Ohio, and shipped alive to Ithaca. These latter animals were larger than the specimens taken locally, ranging from 30 to 37 cm. body length, while the sexually mature specimens from Cayuga Lake were from 22 to 30 cm. long. The Lake Erie animals seemed also to be slightly in advance, seasonally, of the Cayuga Lake specimens in point of changes in the interstitial cells—a difference presumably due to a difference in temperature of the two environments. The animals were killed at intervals throughout the year, specimens being sacrificed in every month save February. The numbers of animals by months are as follows: January one, March one, April ten, May eleven (two immature), June seven, July six (one immature), August three, September three, October four, November three, December three.

Several methods of fixation and staining were employed. The two fixers most extensively used were the dichromate-acetic-osmic mixture of Bensley ('11) followed by his acid-fuchsin and

<sup>4</sup> As this work was nearing completion, my attention was called to an article by Champy ('13), who describes and figures interstitial cells in the testes of several European species of urodeles. Champy is struck by the resemblance of the interstitial tissue to the corpus luteum of the mammalian ovary, going so far as to term it a 'veritable corps jaune testiculaire.' His results are largely in harmony with my own findings, although he is led to the statement of certain general conclusions which are manifestly inapplicable to all urodeles. His results, together with the brief comments of Friedmann and Ganfini, on the occurrence of interstitial cells in Urodeles, will be considered in greater detail in the following pages of this article.

methylen-blue staining technique, and the modified Zenker's fluid followed by dichromate mordantage and Weigert staining as recommended by Kingsbury ('11) for the demonstration of lipoids. In addition to the above, portions of the testis were fixed in Benda's and Regaud's mixtures for the demonstration of mitochondria. Regaud's IV B mixture was most used for this purpose, fixation for twenty-four hours being followed by mordantage in 3 per cent potassium dichromate solution for two to three weeks, and staining by the Weigert method. Fixers less frequently employed were the mixtures of Hermann and Flemming, Bouin's picro-aceto-formol, Carnoy's 6-3-1, concentrated mercuric chloride, 10 per cent formol, ordinary Zenker's fluid, Helly's fluid, and copper dichromate-sublimate-acetic. The testis of *Necturus* being an elongated organ, it was ordinarily cut transversely into pieces of 2 to 5 mm. length for fixation. When studies of the organ indicated it possessed cephalocaudal regional differences, testes were fixed entire, at frequent intervals, and sectioned longitudinally in order that such differences might be conveniently studied in a single preparation. Frequently, also, when material was limited, and studies of longitudinal sections fixed with a number of fluids seemed desirable, the testes were cut in halves longitudinally with a sharp razor instead of being fixed entire, a single animal thus affording four portions, from each of which complete longitudinal sections might be obtained. Serial sections were not considered necessary. All sections were cut as thin as practicable; for more detailed studies sections 3 to 6 $\mu$  thick were used.

In addition to this very complete series of *Necturus* material, there has been available for comparative study material from several other urodele amphibians. The series of testes of *Desmognathus fusca* was especially complete, including specimens taken during every month of the year except January. The greater part of this material had been fixed in Hermann's or Flemming's fluid and stained with iron hematoxylin. The *Diemyctylus viridescens* material in the collection represented animals killed in nine months of the year, and included testes of the immature red land phase as well as those of the mature aquatic

viridescent animal. Through the courtesy of Dr. Bertram G. Smith, a somewhat less complete series of testes of *Cryptobranchus alleghaniensis* was at my disposal—a series including immature males as well as sexually mature animals taken in the months of May to September, inclusive. Some material from *Plethodon erythronotus*, *Plethodon glutinosus*, *Spelerpes bilineatus*, *Amblystoma punctatum*, *Gyrinophilus porphyriticus* and an European form, *Salamandra atra*, was also examined, but of none of these last-named animals have sufficient specimens as yet been secured to constitute even a partially complete series. For comparison, however, with conditions in the other animals at similar stages of the spermatogenetic cycle, this material has proved of no little value.

*Structure of the urodele testis*

The testes of urodeles, as of all amphibia, are abdominal in position. They are more or less elongated structures attached to the dorsal body wall by a mesorchium in which are the vasa efferentia, leading from the testis to the vas deferens, as well as the afferent and efferent blood vessels of the organ. The testes of *Desmognathus*, *Plethodon*, and *Spelerpes* are highly pigmented externally; *Salamandra*, *Amblystoma*, and *Diemyctylus* have testes free, as a rule, from pigmentation; *Necturus* has an irregular mottling of pigment splotches, varying with the individual animal, from almost complete pigmentation (rare) to complete absence of pigment (rare). There is apparently, in *Necturus*, no definite relation of pigmentation to period in the spermatogenetic cycle as there is in the deeply pigmented testes of *Desmognathus*, in which pigmentation diminishes with the expansion of the organ, to increase as the organ shrinks during the regressive changes following the completion of the cycle.

The structure of the urodele testis requires detailed consideration, since as has been stated it is an organ exhibiting, in comparison with the testes of higher animals, a much more complete isolation of the different stages of the spermatogenetic process.

Spengel ('76) describes the testis as consisting of 'capsules'<sup>1</sup> opening by means of collecting tubules to a large collecting duct. The lobules vary in their relations to the main collecting duct, and on the basis of this variation, Spengel recognized three structural types. The testis of his first type has a longitudinal collecting duct central in position, around which the lobules occupy a radial position; this type is seen in *Batrachoseps*, *Gyrinophilus*, *Plethodon*, and *Spelerpes*. In this type the lobules are of a fairly uniform conical or pyramidal shape, each with its base extending to the periphery of the organ and its apex lying adjacent to the central collecting duct, to which it opens directly by a single short unbranched tubule. The testis of this type, as might be expected, is cylindrical, though varying in diameter in different portions at different seasons, for reasons which will become apparent later in this discussion. In the second type of organ the longitudinal canal is superficial, and, when seen in a transection of the testis as in figure 3, the lobules appear arranged in the fashion of an open fan, as in *Necturus* and *Cryptobranchus*.

As in testes of the first type, each lobule extends from the periphery to the vicinity of the longitudinal collecting duct; the latter is located in the connective tissue at the hilus, which runs the full length of the testis. In *Necturus* this longitudinal duct has more the nature of a series of enlarged irregular chambers, connected, however, by narrower passages into a continuous chain, from which smaller tubules are given off; each smaller tubule branches irregularly, the terminal branches finally connecting with the apices of the elongated, conical lobules. In Spengel's third type the lobules are described as being more spherical and terminating the numerous divisions of a much-branched collecting duct; here, as may be seen in figure 7, not all the lobules extend from main duct to periphery, but many are entirely peripheral in position, being supplied by long branches running out to them, between the more centrally placed

<sup>1</sup> I shall use in this discussion the preferable term 'lobules' introduced by Kingsbury ('02). He considered them as being, probably and essentially, homologous with the lobules of the mammalian testis, though not differentiated into tubuli contorti in the urodele as in the mammal.

lobules, from the main collecting duct. This type of testis is seen in *Salamandra atra* and *Diemyctylus*; it is due to the elongation of the duct branches referred to above that the organ assumes the more rounded form seen in these urodeles; in *Diemyctylus*, in particular, the transverse diameter of the testis may at times be nearly equal to its length.

Between the three types of structure recognized by Spengel there may be transitional forms. Kingsbury ('02) describes the testis of *Desmognathus* as being a combination of two of these types, the longitudinal collecting duct, central in position through most of its extent becoming peripheral toward the ends of the organ. In passing, it might be mentioned that Hoffman ('78) suggests the homology of this central duct and its branch tubules with the rete testis and vasa recta of higher vertebrates.

Though differing in their arrangement and relation to the collecting duct, the lobules in the testis of all urodeles are fundamentally the same. They are the structural units of the testis. Each, as has been noted, is connected to the central collecting duct, or to one of its larger branches, by a short tubule. Each is separated from its neighboring lobules by an investment of connective tissue, which is much less apparent when the lobules are in their most distended condition. The development of the lobule, as described by Meves ('96) in *Salamandra*, McGregor ('99) in *Amphiuma*, and Kingsbury ('02) in *Desmognathus*, applies, in general, to the lobule of all urodele testes observed in this study. It begins as a group or vesicle of large primary spermatogonia at the end of a cord of cells representing the branch tubule. (The term 'cord' is used because the lumen may not always be evident.) Each primary spermatogonium (fig. 1) has surrounding it one to several cells of epithelial type, forming a 'follicle.'<sup>6</sup> By increase in number these spermatogonia form a hollow vesicle—the young lobule (fig. 2); the walls of this lobule later become made up of groups or cysts of cells, each group representing the product of several divisions of one of the early

<sup>6</sup> The follicle cells of the urodele testis are similar in function to the Sertoli cells of the mammal, and will be referred to frequently in this paper as Sertoli cells.

spermatogonia, and remaining enclosed in its original follicle whose cells have divided to keep pace with the increasing number of germ cells. The lobule is in addition invested by its sheath or membrane of connective tissue. By the growth and maturation divisions of these cysts of 'secondary' spermatogonia the lobule, now much distended, becomes made up of masses of spermatids, which no longer enclosed in the follicle cells though

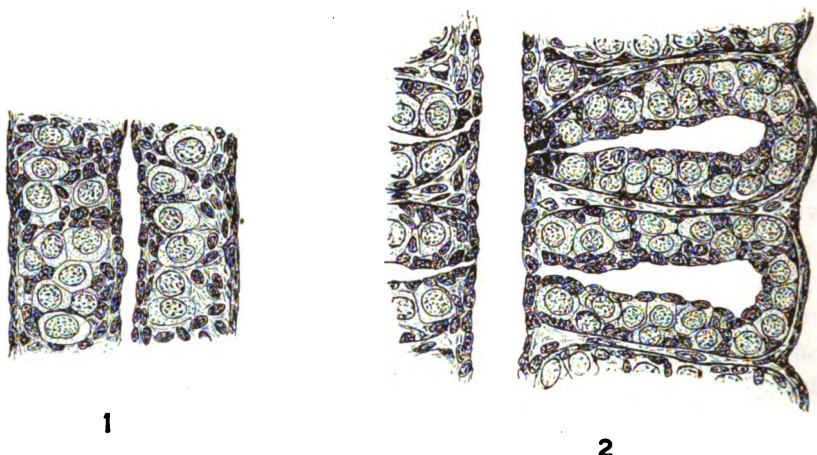


Fig. 1 Longitudinal section of the extreme anterior end of a *Desmognathus* testis. The central collecting duct is shown, bordered by primary spermatogonia with their surrounding follicle cells.

Fig. 2 Longitudinal section of a *Desmognathus* testis, showing a region somewhat posterior to that pictured in figure 1. The developing lobules are here well defined, although containing as yet only primary spermatogonia. Their connection with the central collecting duct is now evident. Only flattened stromal cells appear between the lobules.

in close relationship thereto, undergo the well-known transformation changes, and eventually pass out of the lobule as the spermatozoa.

The development of the lobule needs for the present no more detailed consideration than that given above; for greater detail the various papers on the spermatogenesis of urodeles may be consulted. It should be emphasized, however, that all the cells of the lobule undergo the various developmental changes synchronously, or approximately so; hence the lobule, in successive periods,

will be filled entirely with spermatogonia, spermatocytes, spermatids, or ripe spermatozoa, but will never at any time contain all of these stages, as happens in the mammalian tubuli contorti. Finally, after the spermatozoa have left the lobule, it will contain only the Sertoli cells. These do not persist, but rapidly undergo degenerative changes and are in time absorbed. The degenerating portion of the lobule does not contain developmental stages of the germ cells; these occur in the apex of the lobule, developing as a new structure. Since the only structure common to the two parts is the connective-tissue wall, the emptied portion will be referred to as the emptied or degenerating 'lobule,' while the mass or vesicle of spermatogonia in the apex will be termed the young or regenerating 'lobule.' The former is pushed to the periphery of the testis by the rapid development of the young lobule in some urodeles. This McGregor ('99) states as the case in *Amphiuma*, in which "a testis examined shortly after the breeding time shows a rapid cell proliferation from the center, which is to form the new 'tubules,' while the remains of the old 'tubules,' filled with the debris of degenerating tissue, are seen in the peripheral portion."

A brief discussion of the changes in external form of the testis seems not inappropriate at this point. It is evident that both size and shape of the organ at any time will depend upon the state of development of its component lobules. Urodeles possess a decided cephalocaudal differentiation of the testis; a spermatogenic 'wave,' as noted by numerous investigators, passes from the caudal to the cephalic end of the organ. This 'wave' may be rapid in its progress, in which case there results comparatively little regional differentiation of the testis, as in *Necturus*; it may, on the other hand, be comparatively slow in its movement, in which event regional differences become more pronounced. This latter condition prevails in the testis of *Salamandra maculosa* (Meves, '96), *Salamandra atra*, and *Diemyctylus*, in which at the proper time of year in cephalocaudal succession, may be found 'lobes' (regions composed of few or many lobules) filled with spermatogonia, spermatocytes, spermatids, or mature spermatozoa, with perhaps some of the most caudal lobules



empty and their follicle cells degenerating. The separation of these 'lobes' may be well marked externally, both by intervening constrictions (usually not decided) and color differences. In *Diemyctylus* one may easily recognize the enlargement containing developing germ cells by its transparent blue-gray color; the lobe containing spermatozoa by its pale yellow color. In *Desmognathus* there are similar regional differences, but division into lobes is less apparent. The anterior portion of the testis (fig. 6) in which are only primary spermatogonia, is elongated or thread-like, and the caudal shrunken portion, in which lobules are degenerating, is usually distinguishable because of its reduced size; otherwise there is recognizable no division into lobes. There are, however, in larger *Desmognathus* males, and in *Diemyctylus* and *Salamandra* as well, divisions of the organ which are in structure separate testes, each possessing the differentiation characteristic of the single testis as described above. These divisions, although termed 'lobes' by many investigators, are not to be confused with the lobes previously mentioned. They have been thought of as being of segmental origin. In my opinion they are but the expression of the pattern of growth and degeneration peculiar to the testes of those species in which they occur; since this problem is hardly within the scope of this paper, it is reserved for separate and more detailed discussion in another article.

The caudocephalic progress of a spermatogenetic wave, which results in such distinct lobation in some urodeles, has, because of its comparative rapidity, much less effect upon the form of the testis in *Necturus*. The caudal portion of the organ enlarges earliest in the season, due to its earlier ripening of spermatozoa; it becomes smaller when its spermatozoa are extruded, but prompt regeneration of the lobules brings it back to a condition such that by late winter the testis is of fairly uniform diameter, though tending to taper somewhat toward the anterior end and to present a more blunt or rounded aspect caudally. The structure of the organ at different seasons will be discussed in greater detail in connection with the description of changes in the interstitial cells. Sufficient regional differentiation occurs

so that to some extent successive developmental stages may be obtained for study in a single longitudinal section. The wave of spermatogenesis is clearly much more rapid, however, than in *Desmognathus*, *Diemyctylus*, and *Salamandra*, tending to cause the germ cells of all parts of the testis to be in more nearly the same stage of development at any particular time.

Regardless of the arrangement of the lobules in the testis and the varying rapidity of the degenerative and regenerative changes they undergo at the close of the spermatogenetic cycle, the testes of all urodeles examined show, in common, this fundamental feature: the lobule develops as a unit; its germ cells all mature and leave the testis as spermatozoa, after which the lobule, more or less as a unit, degenerates and disappears. And since the developmental and degenerative processes of the urodele testis are widely separated in this way in point of time, only one spermatogenetic cycle being completed each year, or isolated regionally in the organ through the cephalic movement of a spermatogenetic wave, we can turn to a study of these lower forms to more clearly demonstrate with which phases of spermatogenesis, if any, interstitial cell development may be most closely correlated.

#### *Spermatogenetic cycle and mating period of Necturus*

*Necturus* males killed in November and the winter months following show few or no spermatozoa in the testis. Those present occupy a few lobules in the most cephalic portion of the organ. They represent the last-matured germ cells of the recently ended sexual cycle, and entirely disappear from the testis before the end of the following April. During the winter months primary spermatogonia occupy the apices of the lobules, which are now partially filled with secondary spermatogonia. Cell divisions may be seen during these winter months, spermatogenesis evidently proceeding, however, at a much reduced rate in winter as compared with later spring months. In the spring and early summer rapidly succeeding divisions result in the secondary spermatogonia occupying the entire testis by July.

The period of growth follows; maturation divisions occur in July and August, and in the latter month transforming spermatids are present in the caudal lobules. By October spermatozoa are mature throughout the testis; animals killed late in the month showed the greater portion of the testis empty and the caudal lobules well advanced in degenerative changes, while the spermatogonia were growing to produce the lobules of the next sexual cycle. The mating period presumably occurs in the autumn at the close of the spermatogenetic cycle, since it is at that time that the greater part of the spermatozoa are extruded from the testis. The spermatozoa present in the spermathecas of the female in the fall and winter months (Kingsbury, '95) are doubtless acquired in autumn rather than held over from a spring mating, if such should occur.

#### *Interstitial cell cycle in Necturus*

*a. Absence of such cells in summer months.* During July and August one finds throughout the testis the spermatocytes in various growth and maturation stages, with transforming spermatids in a few caudal lobules of the testis perhaps before the end of the last-named month. The testis, during these months and in September, is at the greatest size it attains at any time in the sexual cycle. The increase is in the diameter more than in the length of the organ. The individual lobules retain their customary fan-shaped arrangement about the superficial longitudinal collecting duct; each lobule extends from the hilus, in which this duct lies, to the periphery of the testis. The lobules, by their increased length, have brought about the increase in diameter so noticeable at this period, and though each elongated lobule will now contain germ cells in some one of the late stages of spermatogenesis, in the centrally located apex of each lobule, as shown in figure 3, will be found the primary and even some secondary spermatogonia from which later will develop the regenerated lobule of the next sexual cycle. The elongated lobules during this period are greatly distended, so that they come to press closely one upon the other; their shape is modified

accordingly. A tangential section of the testis, as in figure 8, shows the lobules in cross-section as several-sided polygons rather than as circles, with the blood vessels occupying spaces where angles of three or more polygons join. The lobules, then, are at this time pyramidal rather than conical, and fit quite closely together, with the result that the interlobular tissue or stroma is subjected to considerable compression, if appearances of fixed material are at all indicative of conditions in the living animal.

Careful study of the interlobular tissue at this period (July, August) reveals as a rule no cells recognizable as interstitial cells by the usual criteria (i.e., rounded nucleus, polyhedral cell form, plentiful cytoplasm with lipid or other accumulations). Between the lobules, either as they are cut in cross or longitudinal section, appear nuclei of an elongated form, (figs. 9 and 25) from 25 to 35  $\mu$  in length, and 5 to 8  $\mu$  in thickness. From the fact that they are elongated when seen in either of these planes, one would infer that they are of a flattened or disk shape rather than of the true spindle type. This is confirmed by seeing them in a surface view where a lobule chances to be cut tangentially; they then appear either circular or broadly oval with their diameter then approximating the 'lengths' observed for the elongated nuclei. These nuclei contain numerous coarse masses of chromatin, which stains more deeply and holds its stain more tenaciously than the chromatin of the germ cells; no nucleoli are apparent. There are no definite cell boundries to be detected between adjacent nuclei. In some cases, a small amount of cytoplasm can be seen at one or both ends of the nucleus; any cytoplasm present must be flattened out between lobules as are the nuclei. And finally, though the lobules themselves may contain considerable fat blackened by osmic acid, such blackened droplets are not seen in the interstitial tissue in constant, definite association with these nuclei; being, indeed, rarely seen there at all. The application of a mitochondrial technique indicates the presence of occasional fine granules in the cytoplasm; but after fixation with Bensley's fluid, and staining with acid fuchsin and methyl green, the larger fuchsinophile granules so numerous in

later stages do not appear. The granules present are presumably mitochondria, and will be referred to as such.

It was, doubtless, from a study of the testes of *Triton cristatus* and axolotl at periods such as this that Friedmann ('98) came to the conclusion that no interstitial cells were formed in these two urodeles, he stating that only ordinary connective tissue could be found between the lobules. That a study of these species in a different season of the year might have caused him to modify these conclusions would appear from the work of Champy ('13) and Ganfini ('02). The latter described and figured 'granular cells' in the testis of *Triton cristatus*, but his work is somewhat inconclusive, as he does not state when and in what part of the testis these cells appear. Champy undoubtedly observed the interstitial cells in both triton and axolotl, and recognized the fact that they do not appear between lobules during the progressive phases of spermatogenesis. One would certainly seem justified in concluding that no interstitial cells existed in *Necturus* if he limited his observations to the period during which the maturation divisions and the transformations of the spermatid are taking place; neither would he be able to detect them in the testes of *Cryptobranchus*, *Plethodon*, *Diemyctylus*, *Spelerpes*, and *Gyrinophilus*, nor in *Desmognathus* except in the caudal shrunken portion of the testis in which spermatozoa had matured in the preceding summer. To the fact that interest in the urodele testis has centered chiefly about this maturation period and its all-absorbing problems may be ascribed the failure of the majority of investigators to note that in a later season interstitial cells become extremely numerous and prominent. Their presence in the urodele testis, however, is rarely mentioned by workers on the problems of spermatogenesis.

b. *Differentiation of interstitial cells; their mitoses.* In September and October spermatozoa are maturing and leaving the lobules. During the period when the lobules are still distended with transforming spermatids, the interlobular tissue remains in the condition characteristic of the preceding months. With the formation of the spermatozoa, however, the balance of metabolic conditions within the testis is disturbed; physical condi-

tions, such as the intralobular pressure, are likewise altered; profound changes follow. Even before all the spermatozoa have yet left them, the diminution of the intralobular pressure causes the lobules to appear, in cross-section, somewhat less angular than previously, with the interlobular tissue increased in prominence, as is seen in figures 10 and 11.

The interlobular nuclei present in previous months, compressed greatly by the distention of the lobules, now begin to round out. Numerous transitional forms are seen. The flattened disks become thickened, typical nuclei now being from 18 to 24  $\mu$  in diameter and 9 to 12  $\mu$  in thickness. These finally are seen as spherical nuclei of from 15 to 18  $\mu$  in diameter. Figures 26 to 30 illustrate stages in this transformation. The cytoplasm increases also as the nucleus rounds out, and fuchsinophile granules and lipid droplets are soon apparent. These transforming interlobular or stroma cells will henceforth be referred to as interstitial cells, since their succeeding stages leave no doubt as to the correctness of their interpretation as such. It is these cells, doubtless, that Champy has described as forming a 'corpus luteum of the testicle,' since he states that this develops around emptied lobules at the close of the spermatogenetic cycle.

The increase in quantity of the interlobular tissue is not due entirely to the apparent increase which would result from a shrinkage of the lobule, both in length and diameter, as the spermatozoa being to leave it. Neither is it due entirely to increase in the size of the cells present earlier in the summer. Both the above factors operate to bring to any particular interlobular position a greater bulk of tissue. A third factor, however, contributes; I refer to cell division. The flattened nuclei of the July and August period evidently are not under favorable conditions for multiplication, as evidence of only one or two mitoses was seen in these months. The study of the interstitial cells of late October was made before any mitoses whatever had been discovered. The great number of cells then present surrounding cross-sections of lobules, as compared with the few widely separated cells seen earlier, suggested that divisions must occur. (Compare figures 8 and 12.) Search for mitoses among the

cells with rounded nuclei and much cytoplasm proved fruitless; neither could satisfactory evidence of amitosis be secured. Finally, turning to an intermediate period, I found that in animals killed September 25th and October 7th and 21st, in the caudal portion of the testis, between lobules then occupied by mature spermatozoa, mitoses were to be frequently found among those nuclei just beginning to thicken or become rounded. Figures 10, 11, and 27 show such mitoses. It would appear, then, that the most favorable time for mitosis is before the cell has become burdened with lipoid accumulations and increased cytoplasmic content. More rarely divisions are found in later periods; one each was observed in animals killed October 30th, December 10th, and December 12th. In each of these cases the mitotic figure was found in a group of lipoid-filled cells, the accumulations in the dividing cell being pushed to one side, away from the spindle. In no material killed in later winter or spring months have mitoses been noted. That mitoses are most to be expected in the earlier growth period of the interstitial cells would appear from the fact that mitotic figures are found to occur regularly in corresponding regions of the testis in *Salamandra atra*, *Cryptobranchus*, and *Desmognathus*, while in none of these three forms were mitoses seen at earlier or later periods, i.e., in regions of the testes where conditions in *Necturus* had resulted in an occasional cell division.

Mitotic division of the interstitial cells in adult mammals is of infrequent occurrence. B. M. Allen ('04) found mitoses in the interstitial cells of pig embryos "up to the stage of the 7.5 cm. embryo, and in the rabbit testis as late as eight days after birth." Both Allen and Whitehead ('04) found no mitoses in older pigs; Duesberg ('18) found none in his extensive studies on the opossum, though Jordan ('11) reports seeing them in this animal; Rasmussen saw none in the woodchuck. Though several workers—Hansemann ('95), Reinke ('96), Von Lenhossek ('97), and Pick ('05)—have found mitotic figures in adult human material, other workers comment upon their absence, Kasai, among these, seeing but one mitotic figure in a series of 130 human testes—the youngest from a four months' fetus, the oldest

from a man of eighty-four years. The occurrence of interstitial cell mitoses in numbers in any mammal, then, seems limited to its embryonic stages or early postnatal life, as compared with the periodic (annual) multiplication of these cells in the urodeles. It is suggested that in the testes of young mammals a cessation of growth of the seminiferous tubules, or possibly degenerations occurring within them, must furnish conditions favorable to interstitial cell differentiation and multiplication similar to those in the urodele testis at the close of its spermatogenetic cycle. Allen, indeed, has stated that the interstitial cells in embryos are "formed contemporaneously with the appearance of fatty degeneration in both peritoneum and seminiferous tubules;" Whitehead, however, believes that they appear before any such degeneration occurs. The latter, in describing in pig embryos the development of interstitial cells from intertubular tissue of mesenchymal structure, states that mitoses are seen in the early stages of their differentiation. From a similar interlobular tissue in the urodeles—a tissue with a syncytial arrangement and scant cytoplasm, typically a mesenchymal structure—the interstitial cells of urodeles differentiate, with numerous mitoses marking the earlier stages of their transformation. The close time relations of multiplication to differentiation from a connective-tissue type of cell is the same in the embryonic mammalian testis as in the testis of the urodele, and it seems not unreasonable to suspect the operation of fundamentally similar metabolic conditions in both at the time these occur. As noted above, Allen and Whitehead disagree as to what these conditions may be in the embryonic testis, Whitehead reaching the conclusion that "the hypothesis which attributes the growth of Leydig's cells to fatty degeneration in these situations is incorrect;" in the urodele testis the favoring conditions are clearly those resulting from the beginning of regressive changes in the lobules at the close of the sex cycle.

*c. Condition in late fall and winter months.* As the spermatozoa mature first in the caudal portion of the testis, and first leave that portion, it is there that the differentiation and mitoses of the interstitial cells begin, as has already been stated. As the wave



of spermatogenesis moves toward the cephalic end of the organ, leaving behind it a trail of emptied lobules at the close of the cycle, the development of interstitial cells follows in its wake. It cannot be said that at a certain time of year these cells are in a certain condition; there exists in the testis, from late October to the following spring, a succession of stages in their development corresponding with the caudocephalic succession in which the lobules were emptied, the youngest stages present being most cephalically located and the oldest caudally, with intermediate stages between the two. Hence a study of complete longisections of the testis (rather than of cross-sections through any one region) is necessary if one wishes to determine the extent of development of the interstitial cells at a given time.

A longisection of the testis of an animal killed on October 30th shows that the anterior portion (about a third) of the organ is still occupied by spermatozoa. This anterior third of the testis is somewhat larger than the caudal portion, which has shrunk to about two-thirds or less of its previous diameter following the emptying of its lobules. Each sperm-filled lobule is distended so as to press upon its neighbors; cross-sections are polygonal in shape. The interstitial nuclei are as previously described for lobules in such a stage; they have become somewhat thickened, but are still disk shaped, with occasional small spherical mitochondria in their scant cytoplasm.

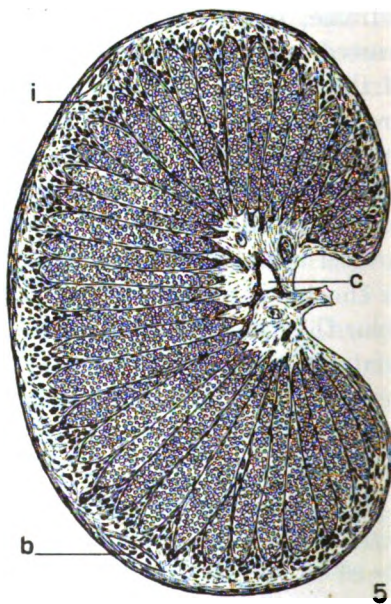
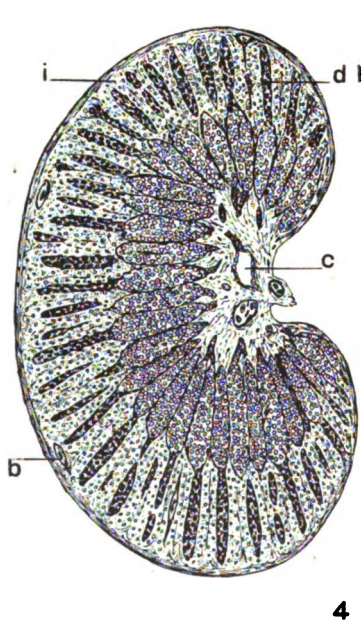
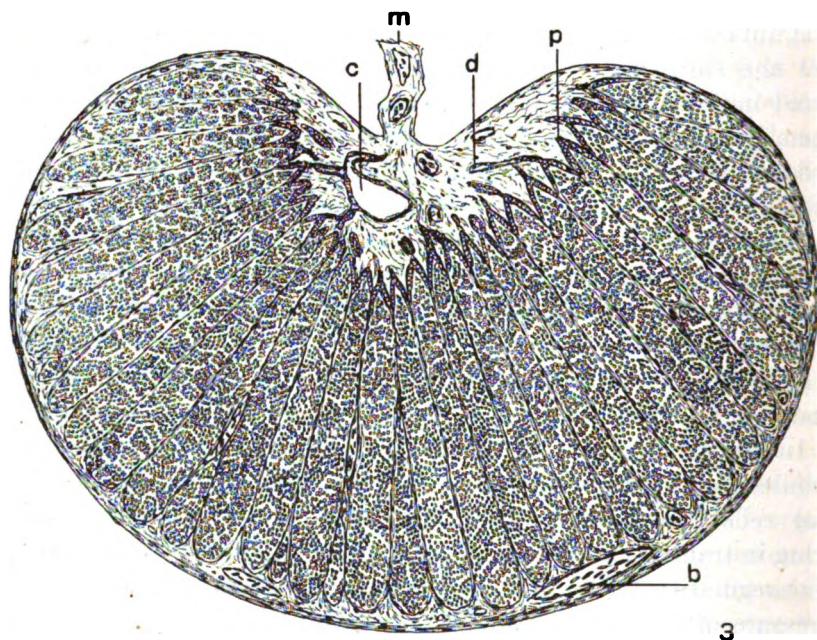
The region immediately caudal to this shows the lobules which have been but recently emptied. In these there is evidence of rapid degeneration of the Sertoli cells, which have now completed their period of usefulness. Nuclei pass through the usual degeneration stages, finally to undergo solution and disappear. Fat demonstrable with osmic acid appears within the lobule. Though it may be present in small droplets in the lobule during the summer, the total quantity appearing then is comparatively small; now, however, the lobule becomes crowded with lipid droplets of various sizes.

Around such degenerating lobules the flattened nuclei rapidly round out, their cytoplasm increases in quantity, and cell boundaries soon become distinguishable. These transformed cells at

first invest the lobule in a sheath one cell layer in thickness; but the shrinkage of the lobule in all its dimensions and the rapid increase in bulk of the cells themselves result in some of them being pushed out, until the sheath becomes two or more cells in thickness. The sheath maintains its position about the lobule. It is seen, in a cross-section of the lobule (fig. 12) as an 'epithelioid ring' of varying thickness. Lobules with their surrounding rings do not now press against each other. On the contrary, each maintains its rounded outline, the interlobular spaces outside the interstitial cell sheaths now being filled with a loose meshwork of connective tissue, in which are blood vessels and 'fixed' connective-tissue nuclei.<sup>7</sup>

In the caudal third of this same testis (of October 30th) the lobules are found to be much more reduced in size and the interstitial cells increased. Each lobule has its surrounding sheath (ring in transection) of two, three, or even more cells in thickness. The regularity of the rings now tends to become obscured by the pressure of one against another; groups of the cells, also, tend to break away from the thickened rings and occupy the spaces earlier filled only by the loose connective tissue. This, in its extreme, results in cords and irregular masses of cells as contrasted with the symmetrical epithelioid rings of the middle of the testis. The cells, 35 to 45  $\mu$  in their greatest diameter, are irregularly polyhedral with round nuclei, and contain numerous fuchsinophile granules and lipoid droplets, which will be considered in greater detail presently. The degenerating lobules and interstitial cells have, of course, been pushed somewhat toward the periphery by the growing lobules, and occupy a place in the testis similar to that indicated in figure 4. The yellow color they now give this region in freshly cut testis is in decided contrast to the pale bluish-white color of the central portion occupied by the lobules of growing spermatogonia.

<sup>7</sup> The term 'fixed' may be applied only rather guardedly here, since one frequently sees among these, cells with more or less resemblance to the cells of the lobule sheath. The difference between cells which remain 'fixed' and those which assume the interstitial cell type is probably not so much a difference in the nature of the cells themselves as in the conditions they have encountered.



The wave of spermatogenesis, then, has brought into existence in the testis, at one time, a complete series of stages in the development of the interstitial cells; one can, by going from the cephalic to the caudal lobules, see the complete history of their development from stromal cells. In addition, the related changes in the lobules can be seen at a glance. As some of the most anterior lobules, the number varying with the individual animal, may contain spermatozoa until March or early April, the testis at any time between October and the succeeding spring shows a similar succession of stages when examined in longisection. The portion of the testis occupied by spermatozoa is of course gradually reduced, with a corresponding cephalic shifting of any particular stage of development of the interstitial cells. As long as the spermatozoa remain in the lobule and the Sertoli cells do not undergo degeneration, there is no development around the lobule of the enlarged interstitial cells. When the spermatozoa leave a lobule, however, and degeneration within it begins, the surrounding cells then become modified accordingly. The modification, then, occurs always in relation to changes within the lobule—changes which can readily be seen to be regressive, since the contents of the lobule undergo degeneration and disappear from the testis.

That the interstitial cells are in any way concerned with the growth processes of the new spermatogenetic cycle is not evident. The developing lobules of spermatogonia during the winter months grow out from the hilus or region of the longitudinal

Figs. 3, 4, and 5 Cross-sections (diagrammatic) of the testis of *Necturus*, to illustrate the seasonal variations in its size and structure. All  $\times 11$ .

Fig. 3 The testis as it appears in August. The lobules are distended with spermatids. No interstitial cells are present at this period. *c*, longitudinal collecting duct; *d*, branch of the collecting duct; *p*, primary spermatogonia at lobule apices; *m*, mesorchium; *b*, blood vessel.

Fig. 4 The testis (caudal portion) in late December. The regenerating lobules contain primary and secondary spermatogonia, while the peripheral half of the testis is occupied by degenerating lobules and interstitial cells. *d.l.*, degenerating lobule; *i*, interstitial cells. Other abbreviations as above.

Fig. 5 Testis in June shortly before the final disappearance of the interstitial cells, which at this time are seen as scattered hypertrophied cells at the extreme periphery of the organ. Abbreviations as above.

collecting duct toward the periphery of the testis (figs. 4 and 5). During such growth in *Desmognathus* there are no interstitial cells present, since in this urodele as well as in *Salamandra atra* and *Diemyctylus* lobule regeneration is delayed until after complete disappearance of interstitial cells from the region. The lobule development clearly could not, therefore, in these urodeles affect directly the interstitial cells or be affected thereby. Though in *Necturus* the interstitial cells are still present, whenever they chance to be included between the outer ends of adjacent growing lobules they either degenerate or revert to the stromal cell type from which they arose, never persisting long as interstitial cells between lobules of spermatogonia (figs. 15 and 16). This condition is quite the contrary of what one would expect if they were to serve the 'trophic' function ascribed to them by some investigators (e.g., Plato, '97).

The modification of stromal into interstitial cells, therefore, is clearly a temporary response to conditions within the lobule; when such favoring conditions no longer prevail, the original cell type may be resumed or the cells may degenerate, the proximity of the regenerated lobules in *Necturus* apparently hastening these processes.

A peculiar aspect of the modification of stromal into interstitial cells is to be noted in the anterior end of the testis, in which spermatozoa may remain in some animals as late as April. When the lobules are finally emptied or the spermatozoa within them undergo degeneration, the stromal cells surrounding each lobule begin their usual changes; and, in the case of the lobules earlier emptied, may form a sheath of enlarged cells one cell layer in thickness. The tendency, however, is for cells surrounding these late-emptied lobules to undergo comparatively little change, the majority of them never progressing beyond the earlier stages of modification. In May, instead of a complete ring of interstitial cells around cephalic lobules, one finds perhaps two or three enlarged cells laden with fat droplets, as in figure 13, the other surrounding stromal cells have the flattened form and few or no fat droplets. That mitoses in this region have been suppressed also may be inferred from the comparatively small number of

cells around each lobule. Between the cephalic and caudal lobules intermediate ones present a series of intermediate types; even in the middle of the testis interstitial cell development does not reach the limits set in the most caudal region.

It is suggested that the explanation of the above lies in the modifications in this region of the conditions under which the interstitial cells develop. The caudal lobules are emptied early in the fall; their degeneration begins at a time when the stream of nutritive material no longer used by the reproductive cells is still being brought to the testis, undiminished by any decreased metabolism of the animal due to the onset of colder weather. Since the lobules of the next sexual cycle are as yet little developed, there is but slight opportunity for them to affect, either by mechanical pressure or diversion of the nutritive material, the modification of the stromal cells around the emptied lobules. The cephalic lobules, which are emptied more slowly during the winter months, undergo their degenerative changes during the period of the animal's lowest metabolic activity. The developing lobules of the anterior end of the testis are but little behind those of the caudal end; and, before the spermatozoa have left all the anterior lobules, these regenerated lobules are of such size that they very possibly affect, both by their growth pressure and demand for nutritive materials, the course of development of the interstitial cells around the degenerating lobules. Though factors other than those named may contribute to the difference, the reduced number of interstitial cells of the cephalic region stands at any rate as the expression of a less favorable environment rather than as an indication of intrinsic differences of the stromal cells of the two regions. That those of the cephalic region possess the same potentialities as those in any other is evidenced by the changes many of them undergo.

*d. Details of cell structure.* The finer details of cell structure in successive developmental stages require a more extended description than that previously accorded them. In cells around recently emptied lobules (or even earlier) fuchsinophile granules of a size considerably larger than the minute mitochondria appear (figs. 27 and 28). Though the mitochondria are also

stainable with fuchsin, they do not hold their stain so tenaciously as do these granules. The latter, though more easily preserved than mitochondria, are similarly destroyed by fixation in alcohols, or Bouin's fluid (picro-aceto-formol), and poorly preserved in Zenker's fluid with the usual percentage of acetic acid. They may be demonstrated excellently by Regaud's fixation and subsequent staining by the Weigert method. With this technique, they are much more resistant to differentiation than are the mitochondria in adjacent spermatogonia of the same section, remaining a deep black after treatment with the borax-ferricyanide differentiator has rendered the mitochondria colorless. The mitochondria of the germ cells are, similarly, not well demonstrated in Bensley preparations in which these granules are excellently preserved and intensely stained. The suggestion is strong that they differ sensibly from mitochondria, presumably by having a larger proportion of lipoid in their make-up.

It is found after osmic-acid fixers that the granules do not usually blacken as do the larger lipoid droplets. They are more perfectly preserved than the latter by such fixers as Regaud's or the modified Zenker's. This less perfect preservation of the larger droplets may, indeed, be ascribed to their greater size and fluidity; the failure of the granules, however, to blacken with osmic acid suggests some chemical difference as well and the greater activity of their substance in reducing dichromates bears out this suggestion. The greater solubility of the larger lipoid droplets is apparent after osmic-acid fixation (Bensley's). In stained preparations mounted with cover-glasses the blackened droplets may be entirely dissolved while the fuchsinophile granules appear undiminished in numbers.

That the fuchsinophile granules arise from the smaller mitochondria might be inferred, since they appear gradually in cells in which mitochondria are already present, and upon first appearance are of much smaller size than they subsequently become. Later, too, when they are maximal in number as in figures 29 and 30, sizes intermediate between the largest granules and the mitochondria may be distinguished. Other than this, I have secured no evidence of their mitochondrial origin. The granules of inter-

mediate sizes resemble the larger in their fuchsinophile nature. I have not succeeded in staining them and the mitochondria differentially, though the latter do, of course, retain their stain less tenaciously.

Several attempts to stain the mitochondria *intra vitam* with janus green after the method of Cowdry ('16) proved unsuccessful. Mitochondria were faintly stained, if at all, and other structures, such as some of the larger granules or even lipid droplets, took the stain, appearing to have a film of stainable substance at their periphery. The specificity of janus green as a mitochondrial stain would appear, from my results, somewhat questionable; Bensley ('11) similarly describes it as staining small granules in the islet cells of the pancreas as well as the mitochondria of those cells.

Even as the origin of the larger fuchsinophile granules from smaller mitochondrial ones is suggested by their sizes and order of appearance, so, too, is there a suggestion that these granules may in turn give rise to the droplets which blacken with osmic acid. The smallest of these droplets in Bensley preparations are often of the same size as the larger fuchsinophile granules (figures 29 and 30); frequently blackened droplets seem to stain with the red as well; the droplets usually appear first in that region around the centrosphere in which the granules are most numerous; with the increase in numbers of the droplets, fuchsinophile granules may be much less numerous—in some cells, indeed, almost none at all may remain. •

The lipid droplets blackening with osmic acid appear early in the transformation of the stromal into interstitial cells, some often being seen before the nuclei have assumed the rounded form. They increase in size, some attaining diameters of 6 to 8  $\mu$ ; they increase in number as well, until the cell, except in the region of the centrosphere, may be fairly packed with them, the fuchsinophile granules then appearing much less numerous.

The lipid droplets of the interstitial cells, after osmic-acid fixers, are much more difficult to retain than are those in the degenerating lobules. The latter droplets are retained, as a rule, in covered sections from which the interstitial cell lipoids are



dissolved either wholly or in part. The lipoids of the degenerating lobules do not (except for a short time immediately after the emptying of the lobule) reduce potassium dichromate sufficiently to be preserved by modified Zenker technique, which demonstrates the interstitial cell lipoids, though without preserving perfectly the form of the droplets. After staining with copper or iron hematoxylin, material thus fixed shows the degenerating lobules entirely unstained while the interstitial cells are deeply colored, due to their chromated lipoids.

No chemical analyses of the testis were made by the writer, but from the characteristics of these lipoids it may safely be assumed that they are similar to the phosphatid lipoids and cholesterinesters described by Whitehead ('12), Hanes ('11), and other workers as occurring in the interstitial cells of mammals.

Neither intracellular masses of secretion product (other than the fuchsinophile granules and lipid droplets already described) nor accumulations of such a product in spaces between cells have been encountered in my material. Such intercellular accumulations have been reported by Lenhossek ('97) for man, Senat ('00) for the rat, Bouin and Ancel ('03, '04) for the fetal horse, and Duesberg ('18) for the opossum. Duesberg describes an intercellular space filled with a secretion substance from which he could see, on the one hand, processes extending back into adjacent cells and, on the other hand, a connection with a small capillary. This is surely, as he says, "one of the clearest instances in which the secretion product of a gland with internal secretion actually could be followed from the glandular cell into the vascular system." Had there been present in *Necturus* such prominent masses as he and other workers describe, they would doubtless, after some of the great number of fixers and stains I have employed, been easily detected. Degeneration products, which I mention subsequently, are seen in the cells, but could not be mistaken for a product of regular glandular activity.

Crystals, as described by Reinke ('96) in the interstitial cells of man, and by Duesberg ('18) in the opossum, have not been encountered in *Necturus*. Neither do I find pigment, such as Rasmussen ('17) describes in the woodchuck. The region occu-

pied by interstitial cells is in a freshly cut testis, particularly in spring, of a yellow or orange color, but this seems due to coloring matter in the lipoids, since no pigment is found after fixation.

A peculiar network frequently appears, often filling the entire cytoplasm. Its trabeculae and meshes vary greatly in size in different cells. This network has the same staining reactions as the fuchsinophile granules. It appears best, as a rule, after Bensley's Benda's, or Flemming's fixation, but may be seen also after Zenker's with high percentage of acetic acid. Since the individual fuchsinophile granules are not seen in cells when the net is present and since the network is not seen in the cells of the outer part of the tissue block where fixation of granules is most perfect, I have concluded that this feature is a fixation artifact rather than a characteristic cell structure. This is borne out by preparations with well-fixed peripheral cells. Between such cells and the centrally located ones with networks appear transitional forms. First are cells with only a few of the granules fused, then cells with a partial net and perhaps half of their granules well preserved, and finally cells in which only a very few granules retain their identity. The explanation of this clearly lies in the slow penetration of the osmic acid in the fixers named. This permits the more rapidly penetrating acetic acid to reach the interior of the tissue block first and there act alone upon the fuchsinophile granules, which are particularly sensitive to it. They appear to undergo solution and spread out into the cytoplasm; droplets thus come in contact with adjoining ones, and an irregular net results. The poorly preserved lipid droplets dissolve out in embedding or in staining the sections, and the open meshes of the net result. Particularly coarse trabeculae are seen in regions where the granules are most numerous and fat droplets few and small; in the spring in cells which have become filled with lipid droplets, among which are but scattered granules, the net produced has correspondingly fine trabeculae and open meshes.

A similar network has been described by Duesberg ('18). Like myself, he considers it a fixation artifact, but states that it

arises from poorly preserved mitochondria, which, according to his description and figures, are very numerous in the interstitial cells of the opossum. In *Necturus*, however, the fuchsinophile granules must be responsible for the artifact, as it never occurs prior to their being present in considerable numbers. In the primary spermatogonia of *Necturus*, moreover, in which mitochondria are particularly numerous, I have not been able to find such an artifact, even in sections showing it best in the interstitial cells.

The granules and lipoid droplets, from their early appearance, are not distributed uniformly throughout the cytoplasm. In cells cut in the proper plane, as in figures 28 and 30, a small area at one side of the nucleus appears free from granules, which, however, are often far more numerous at the immediate periphery of the area than in other parts of the cell. The nucleus, as soon as cell boundaries are distinguishable, is seen to lie at one side or end of the cell; the area mentioned, clearly an idiozome, lies on that side of the nucleus toward the center of the cell. In early stages this idiozome is relatively very small, and centrioles are difficult to distinguish except when seen during mitosis, at which time they stain sharply. As the cells increase in size, however, the centrosphere becomes greatly enlarged. In the cells of the caudal third of the testis, on October 30th, are centrospheres from half to three-fourths the diameter of the nucleus. The material composing them is gathered about a distinct centriole as a center; two centrioles may be seen occasionally. It often appears to differ from the other cytoplasm of the cell in its tendency to stain faintly with the basic stain. Immediately around the centriole there appears a small area of rather dense homogeneous material, outside which are faint cytoplasmic radiations extending to the periphery of the centrosphere. In earlier stages there is no sharply defined boundary between the centrosphere and the surrounding granules, but in the spring months centrospheres frequently appear to have a peripheral zone of more dense material inside which the granules are not seen (fig. 31). The centrosphere is then more clearly delimited. In a few of the most extreme cases this peripheral zone has the appearance even of a distinct membrane.

Child ('97) has called attention to the occurrence of similar giant centrospheres in the ovarian stroma (interstitial cells) of the dog and rabbit. Allen ('04) pictures them also in his figures of the interstitial cells of the mammalian testis and ovary. Champy ('13) describes them in the interstitial cells of the European tritons and axolotl. Their occurrence in interstitial cells, therefore, is probably more or less common.

Such giant centrospheres have been very carefully studied by W. H. Lewis ('20), who finds them of frequent occurrence in living mesenchyme cells of tissues cultivated in vitro. He found that cells containing them die sooner than the more normal cells, and concluded that their presence may be regarded as a degenerative change. In my material, likewise, the giant centrospheres are seen in the older rather than the younger, more active cells. The arrangement of fuchsinophile granules and lipoid droplets around them suggests the action of the centrosphere as a center of metabolism—a dynamic center of the cell; nevertheless, the accumulation of such material largely occurs before the centrospheres attain their maximal enlargement. Though the enlarged condition doubtless indicates a regressive change, it does not seem, in my material, to represent an irreversible degeneration. Cells possessing giant centrospheres become included between growing lobules of spermatogonia, where they may be seen with their granules and lipoids partially absorbed and the centrospheres more or less reduced, while their nuclei, still normal in appearance, are more or less flattened. Some of these unquestionably revert to the stromal cell type; other cells whose regressive changes have been more pronounced, may not be able to do so, as the large number of degenerations late in the season indicates (figs. 18 and 33).

*e. Final degeneration or reversion of the cells.* The growth of the regenerating lobules, which has proceeded slowly during the winter, is greatly accelerated by the more favorable conditions of the warmer spring months. As the lobules extend farther and farther toward the periphery of the testis, the degenerating lobules and interstitial cells come to occupy a proportionately smaller space in that region. The degenerating

lobules of the posterior part of the testis by this time are very much reduced in diameter; they stand out in osmic-acid preparations, however, because of the large droplets of fat they contain. Though the Sertoli cells within these lobules have largely degenerated, some few of them are found in apparently normal condition late in the spring. The connective-tissue membrane which surrounds the emptied lobules does not degenerate in *Necturus*, but persists, and within this the cysts or groups of spermatogonia push toward the periphery.

During the course of the growth changes, the interstitial cells become closely compressed between lobules which contain rapidly growing spermatogonia (figs. 15 and 16). They earlier become so numerous in the more caudal parts of the testis as to crowd closely together, rendering less apparent their original epithelioid ring arrangement around the lobules. Now, as illustrated by figure 15, they become closely packed between lobules, filling all those spaces which were earlier occupied by the loose connective tissue. The changes that follow are the reverse of those by which they originated. Their cytoplasm decreases in quantity and its fuchsinophile granules and lipoids gradually disappear, while the nucleus changes from a spherical to a flattened form. The cells thus, after a time, are no longer recognizable as interstitial cells; but such a series of stages is present, often around a single lobule, as to enable one readily to follow the transformation. The typical interstitial cells are of course at the periphery, while intermediate types appear in regions which were but a short time previously subjected to the pressure of the growing spermatogonia.

Since an increase in the number of these cells by mitoses, as I have already described, occurs preceding and during their modification, a compensating reduction of their numbers through cell degenerations is to be expected. Degeneration figures, in fact, appear early in the fall in the caudal region among cells which have attained their maximal enlargement. Degenerations may be frequently observed during the winter months as well. In the spring months, however, they become especially prominent. The earlier degenerations were noticeable chiefly through the

nuclear changes; the lipid droplets and fuchsinophile granules present were as in neighboring cells or were reduced in numbers. In several animals killed in April and May, however, the type of degeneration observed is quite different. Many of the interstitial cells now enlarge, as shown in figures 33 and 34, attaining diameters of from 50 to 80 $\mu$ ; in these the nucleus, rounded in the normal type, becomes shrunken and flattened at one side of the cell, frequently appearing very irregular in shape, and sometimes staining with the acid rather than the basic stain. The lipid appears in much larger droplets than heretofore, and after fixation in Bensley's fluid becomes noticeably more resistant to solution than were the lipoids present in earlier stages or, for that matter, those now contained in adjacent interstitial cells not similarly changed from the earlier type. The enlarged centrospheres seen previously disappear from these cells; in a few cases what might perhaps be a remnant of it could be seen as a mass, staining with the basic stain, lying near the eccentric nucleus. There appear in most of these cells masses of varying size and form which stain with the acid fuchsin. These masses are derived from the granules seen previously. In many of the cells there can be no doubt of this, for the mass (as in fig. 33) can be seen to be made up of cytoplasm in which individual granules are yet distinguishable; in one case the granules were grouped around what must formerly have been the giant centrosphere, to judge by its size and staining reaction. In still other cases small blackened droplets were included in the mass with the granules. In many cells, however, the identity of the granules becomes lost and only solidly staining fuchsinophile globules or masses appear. All appearances indicate that the cytoplasm has undergone partial solution, for lipid droplets and granule masses stand out in fixed material with clear, unstainable spaces intervening, presenting quite a different appearance from that of the normal cells adjacent to them in the same section. Cells of this type do not long persist; they are rarely found in the testis later than July.

Through the numerous degenerations of the interstitial cells as well as by their return to the stromal cell type, the number of lipid-containing cells is reduced, by July, to a few scattered

cells between the ends of the lobules at or near the periphery of the testis. These cells are hypertrophied, rounded or oval, with eccentric and often irregular nuclei. They are packed with lipoid of a comparatively insoluble nature; fuchsinophile granules and mitochondria are reduced in numbers. The enlarged centrospheres previously described cannot be seen. These cells resemble the degenerate type already described; they seem, however, to have nuclei of greater vitality, and to disappear more slowly. Although an occasional specimen killed in autumn may possess hypertrophied, fat-laden cells which have doubtless persisted through the summer months, in most animals such cells are entirely lacking in August. In July, however, cells derived from this type are fairly numerous; such cells range from the size and content of the hypertrophied cell down to cells with shrunken nuclei and but a single lipoid droplet or one or two red-stained granules in their scant cytoplasm. Frequently, one finds fragments representing the last stages of the nuclei of such cells. Figures 35 to 39 show such a series of degenerate forms as is mentioned above. With the passing of these cells the testis may be said to contain no interstitial cells; the cells seen only as flattened nuclei between lobules are, of course, capable of again becoming modified at the now approaching close of the spermatogenetic cycle.

*f. The testis in immature males.* Testes of three sexually immature males have been examined. These were all fairly well-grown animals, about 21 cm. in length, in which the wolffian ducts were still small, straight, and empty, though two were examined at a time (May) when the ducts of mature animals are yet full of spermatozoa. The testes show no indication that spermatozoa were formed in the preceding autumn; i.e., there are present no traces of degenerated lobules.

The testes are filled with lobules of secondary spermatogonia. As would be expected after a study of the interstitial cells in the adult, there are between these lobules only cells of the flattened type. Some of these, in one animal, show small blackened droplets, though these are by no means so numerous here as within the Sertoli cells. This same animal possesses a few enlarged, fat-

laden cells in the connective tissue about the primary spermatogonia, where interstitial cells in the adult are never developed. Such cells, however, were not seen in the others. In none of these immature animals were interstitial cells found comparable in position, size, numbers, and contents with these cells in the adult. That testicular degenerations in still younger males may give rise to such cells is not impossible, such cells then disappearing as do the interstitial cells of adults. Examination of numerous younger stages, such as have not been available for this study, would be required to settle this point. Extensive spermatogonial degenerations, in one immature animal, had apparently not resulted in interstitial-cell development.

*The interstitial cells of other urodeles*

a. *Desmognathus fusca*. The presence of interstitial cells in the testis of this urodele was recognized by Kingsbury and Hirsh ('12), who, in their paper on the degenerations in the secondary spermatogonia of *Desmognathus*, suggest the growth of the interstitial cells as a possible factor in determining the inception of such degeneration. No further mention of these cells in *Desmognathus* has been encountered.

Kingsbury and Hirsh outline the spermatogenetic cycle in this Urodele as follows: It "may be said to begin in the fall or late summer after the extrusion from the testis of the spermatozoa formed during that season. During the fall and winter months there is a multiplication of spermatogonia and a tardy growth of the spermatocytes I. . . . In the spring the growth of the spermatocytes begins actively, characterizing particularly the months of March, April, and May, while divisions of the spermatocytes occur in May, June, and July. The transformation of the spermatids into spermatozoa preponderates in August and September."

During the occupation of the lobules by spermatogonia, spermatocytes, and spermatids there are, as under like conditions in *Necturus*, no interstitial cells present between them. Flattened nuclei, which, however, seem to be of more nearly a true



spindle shape, may be seen as in *Necturus*; little or no lipoid appears between lobules even at times when there is much of it within them. Pigment commonly occurs, but apparently in extensions of the branched chromatophores rather than in relation to the interlobular nuclei of ordinary flattened or spindle type.

In August or September the spermatozoa of the most caudal lobules become mature and begin to leave the testis. The lobules become reduced in size; the cells surrounding them now appear larger, less flattened, and more numerous (fig. 21). Though shrinkage of the lobule brings nuclei closer together and thus causes an apparent increase in their numbers, an actual increase takes place as well, for mitotic figures may frequently be seen at this time.

As described by Kingsbury and Hirsh, a number of lobules in the cephalic end of the testis degenerate during the summer; this region marks the 'boundary plane' up to which the lobules mature and discharge their spermatozoa during the current season. Figures 6 and 21 indicate the location of this 'boundary plane.' Cephalad of this the germ cells are held over for development in the succeeding season. The lobules caudad of the 'boundary plane' usually are all free from spermatozoa by February or March, as indicated by figure 6 D, only a very few of the most cephalic, indeed, not being emptied by December (fig. 6 C). The enlarged region filled with spermatozoa which is seen posterior to the 'boundary plane' in September (fig. 6 B) appears in the following winter and spring as a greatly shrunken region now only a third to a half the diameter of that part of the testis anterior to it. This shrunken region is filled with degenerating lobules, each of which is surrounded by its greatly thickened sheath of enlarged stromal cells, now with the usual characteristics of interstitial cells. Lipoid droplets are numerous in their cytoplasm, the eccentric nuclei are oval or rounded, and the cells are of an irregular polyhedral type, tending, however, to remain somewhat elongated. At one side of the nucleus is a more or less rounded mass of cytoplasm surrounded by small granules; this contains the centrioles and appears quite similar to the enlarged centrospheres seen in the interstitial cells

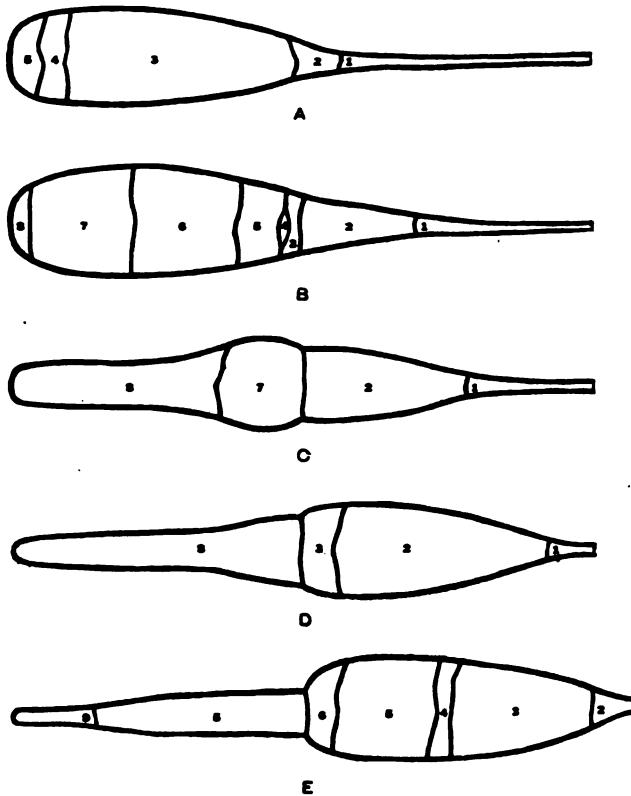


Fig. 6 Diagrammatic longisections of the testis of *Desmognathus* to illustrate the changes, in form and content of the organ, resulting from its slow spermatogenetic wave and delayed lobule regeneration. (A) Early June. (B) Late September. (C) Late November. (D) March. (E) July. The 'boundary plane' in each figure is represented by the line immediately above its title letter. To the left of the boundary plane is the region maturing spermatozoa in the first season; to the right of this boundary plane is indicated the region producing spermatozoa in the following season. Diagrams A and B show the caudocephalic movement of the spermatogenetic wave; diagrams C, D, and E illustrate the changes that follow the emptying of a region. 1, slender cephalic region containing only primary spermatogonia; 2, secondary spermatogonia; 3, spermatocytes I; 4, divisions of spermatocytes I, and spermatocytes II resting or dividing; 5, spermatids; 6, transforming spermatids and immature spermatozoa; 7, mature spermatozoa; 8, emptied lobules degenerating, surrounded by interstitial cells; 9, caudal region from which the interstitial cells have disappeared and only primary spermatogonia appear around the central collecting duct. (Note: The central collecting duct, immediately surrounded by a few primary spermatogonia, extends throughout the length of the testis. Regions 2 to 8 inclusive, therefore, all contain such primary spermatogonia in addition to the elements indicated above.)

of Necturus. The peripheral cytoplasm is filled with larger lipid droplets or vacuolated when these have not been preserved. Figure 22 shows a group of these cells at their maximal size.

The Sertoli cells of the emptied lobules degenerate, lipoids first accumulating within them as in Necturus. Their degeneration proceeds rapidly, so that in the late spring the lobules are practically empty. The residual spermatogonia in the lobule apices, instead of beginning multiplication as soon as the lobules are emptied, or even before, as in Necturus, and so bringing about their prompt regeneration, remain quiescent for several months. As a result of this delay in lobule regeneration, the caudal portion of the testis becomes very much reduced in size, as has been previously stated. After the degeneration of the emptied lobules in this shrunken region, its interstitial cells, though persisting for a time, also disappear. Finally this portion of the testis (fig. 6 E) is reduced to a slender thread-like structure, made up chiefly of the central collecting duct and the residual spermatogonia surrounding it—a structure similar to that shown in figure 1. The primary spermatogonia do not begin multiplication until some time after the disappearance of the interstitial cells; by their growth the lobules of the region are eventually regenerated.

Although such regeneration of the lobules in any region does not occur until after the disappearance of the interstitial cells therefrom, this is more probably a part of a regulated growth plan than a result of any inhibitory influence the interstitial cells may exert. In Necturus, in which regeneration of the lobules occurs more promptly, their growth takes place while interstitial cells are still present in their maximal number. Far from being inhibited by the presence of the interstitial cells, the growth of the lobules, on the contrary, appears instrumental in hastening the disappearance of these elements from the testis. The interstitial cells of *Desmognathus* doubtless owe their longer period of existence to their freedom from such influences. Though tending to disappear when the degeneration of the lobule they surround is completely effected, their disappearance is not

hastened by growth pressures and such other influences as may be brought to bear in an organ full of rapidly developing germ cells.

*b. Diemyctylus viridescens.* The interstitial cell cycle in *Diemyctylus* bears a marked resemblance to that just described for *Desmognathus*, the two differing of course in minor details. The time relations in *Diemyctylus* are somewhat more difficult to determine, especially from examination of but a small number of animals presenting more or less individual variation.

The spermatogenetic cycle may be briefly outlined as follows: The maturation divisions and the transformation of the spermatids are accomplished during the summer months. Some of the spermatozoa leave the lobules in the fall; a mating at that time is known to occur frequently (Gage, '91). The testis in November or December, as in the following spring (fig. 7, showing a longitudinal section of the testis as it appears in May), consists of a cephalic region containing spermatogonia and a caudal region containing mature spermatozoa. The two regions are so separated by a constriction and marked by color difference as to be easily recognized upon gross examination. There may appear at the point of constriction numerous degenerating lobules such as Kingsbury and Hirsh describe in *Desmognathus*; these appear to mark a 'boundary plane' cephalad of which the spermatogonia hold over till the following season.

During the winter the mature spermatozoa remain in the lobules, to be extruded when mating is resumed in the spring. One animal killed May 30th had practically all lobules then emptied, as shown in figure 7, while another killed June 7th had fully as many spermatozoa as animals killed in winter. In one case mating had presumably occurred; in the other all appearances indicated the contrary. Another animal killed June 30th showed maturation divisions in progress, spermatids transforming and spermatozoa yet immature, but no mature spermatozoa of the previous cycle present.

The stromal cells surrounding lobules during the progress of spermatogenesis are of the ordinary flattened type, rarely containing lipoids demonstrable with osmic acid. During the

several months that the spermatozoa remain in the testis numerous lipid droplets may appear between lobules, though the cells do not then, as a rule, round out to a polyhedral form because of the continued pressure of the distended lobules. The appearance of typical interstitial cells, as in other urodeles, occurs only when the lobules have been emptied and degeneration

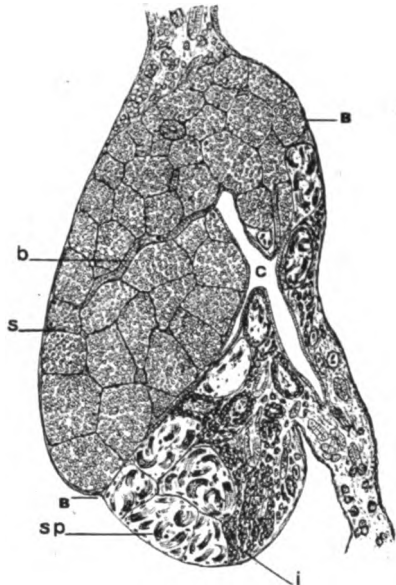


Fig. 7 Longitudinal section of the testis of *Diemictylus* after the spring mating season. Only a few of the lobules still contain spermatozoa. The recently emptied lobules are surrounded by interstitial cells, whose lipoids have been blackened by the osmic acid of the fixing fluid (Bensley's). *c*, central collecting duct; *b*, branch of central collecting duct; *s*, lobules filled with secondary spermatogonia; *sp.*, lobules filled with spermatozoa; *i*, interstitial cells; *B*, boundary plane.  $\times 16$ .

has begun. As this happens in the case of the more caudal ones during the fall mating, there are present during early winter numerous lobules surrounded by well-developed interstitial cell sheaths. The lobule appears to degenerate rapidly; sometimes cords and masses of interstitial cells appear within which but little trace of the lobule remains, although in other cases its connective-tissue wall, thickened, convoluted, and hyaline,

persists even after the interstitial cells have largely degenerated or undergone reversion. That scattered interstitial cells and such traces of degenerated lobules appear in the testis in November and December is indicative of the short time required for the completion of lobule degeneration and the disappearance of the interstitial cells. Two animals killed in January and February show comparatively few interstitial cells present, though in all probability they had been numerous earlier in the winter. There is never a definite, elongated, shrunken, caudal region occupied by interstitial cells and degenerating lobules as in *Desmognathus*.

The emptying of the remaining lobules of spermatozoa during the spring mating period is followed by lobule degeneration and interstitial cell development as in the autumn. Usually the larger number of lobules empty and degenerate in the spring, making the picture a trifle more striking. As in the autumn, the disappearance of lobule and interstitial cells is rapid. An animal killed June 30th had traces of but one or two degenerated lobules still distinguishable and a few scattered interstitial cells; this perhaps represents the extreme of early disappearance. In an animal killed in early August no interstitial cells or degenerating lobules could be found.

My study on this form has not included cytological detail of the cells; they resemble those of *Desmognathus*, though tending, perhaps to possess a greater number of lipoid droplets. The cell form is irregularly polyhedral; the eccentric nucleus is usually somewhat oval; centrospheres have been seen, but not in the enlarged condition noted in *Necturus*. Mitoses were not observed, though an increase in the number of the cells is apparent and suggests their multiplication as observed in *Desmognathus*, *Necturus*, *Salamandra atra*, and *Cryptobranchus*.

It is apparent, I believe, without further description, that in *Diemyctylus* the interstitial cells as in other urodeles are closely related to lobule degeneration. Their early disappearance is of course in decided contrast with their longer cycle in *Desmognathus*. Such a difference, however, only shows more clearly that they have no functional relation to the progressive phases of

spermatogenesis. Never absent in *Desmognathus*, they are entirely lacking during the summer months in *Diemyctylus*, to reappear in the fall, as in *Necturus*, when degeneration of the emptied lobules begins.

*c. Salamandra atra, Cryptobranchus, etc.* Testes of several other urodeles have been examined. Although an account of the interstitial-cell cycle in these must be reserved for a later writing, a brief report of observations on the material already at hand will indicate points of agreement with those urodeles of which more complete studies have been possible.

The testis of *Salamandra atra*,<sup>\*</sup> an European form, shows considerable resemblance to that of *Diemyctylus*. In larger males it consists of two or three enlargements, which, as in *Desmognathus*, are in effect separate testes. The constricted regions between these contain primary spermatogonia ranged around the collecting-duct system. Each enlargement or 'testis' in the material examined consists of two portions of approximately equal size, separated by a slight constriction at which degenerated lobules are commonly seen. In front of the 'boundary plane' thus established, the 'testis' contains only spermatogonia; caudal to it, only spermatozoa or lobules recently left empty through their extrusion.

The cells between lobules of spermatogonia are flattened and without interstitial-cell characteristics. Around the most cephalic lobules of spermatozoa they are somewhat thickened and possess distinctly granular cytoplasm. They are far more numerous here as well. The presence of mitotic figures (fig. 23) indicates clearly their manner of increase. Still farther toward the caudal end of the enlargement or 'testis,' around emptied lobules, interstitial cells appear in rings two or more cells in thickness as shown in figure 24. They are now more or less polyhedral in form; their cytoplasm is most dense at one side of the nucleus, where in a centrosphere free from other granules

<sup>\*</sup> The material loaned me for this study by Dr. B. F. Kingsbury had been collected in Germany some years previously. It had all been taken in the summer months, at the close of the sexual cycle, but no record of dates of killing had been kept.

a centriole is seen; the peripheral cytoplasm appears less dense and is occupied by lipid droplets of larger size. In the most caudal part of the 'testis' the rings of cells become reduced in size and numerous degenerating nuclei appear. Indications are that the degeneration and reversion of the interstitial cells occupy a relatively short period, as in *Diemyctylus*. Champy ('13) states that in the related form, *Salamandra maculosa*, the interstitial cells are likewise temporary. The association of interstitial cells with lobule degeneration and their absence from other regions of the testis are as apparent in *Salamandra* as in other urodeles.

The testis of *Cryptobranchus* is essentially the same in structure as that of *Necturus*. The spermatogenetic wave, however, passes over its entire extent much more rapidly. In early September, for example, the entire length of the testis is filled with spermatozoa, though the caudal lobules may at that time be beginning to empty. This condition is in decided contrast with that in *Desmognathus*, *Diemyctylus*, and *Salamandra*, in which the testis is markedly polar and the wave moves forward over only a part of the organ during any one sexual cycle.

During June, July, and early August the growth of the spermatocytes, their maturation divisions, and the transformation of the spermatids occur in rapid succession. During this period the lobules are greatly distended, and, as in other urodeles, only flattened nuclei appear between them. These are round or broadly oval when seen in surface view, or of spindle form when cut across, as they more frequently are seen. In late August and early September, when the lobules are filled with spermatozoa, occasional mitoses may be seen, even as at a similar period or one slightly later they are common in *Necturus*.

Developments after complete extrusion of the spermatozoa have not been followed. Animals killed in early September either showed no empty lobules or none sufficiently long emptied for interstitial cells to have developed. These observations, therefore, merely show that *Cryptobranchus* agrees with other urodeles in lacking interstitial cells in association with the earlier or progressive phases of its spermatogenetic cycle.



The testis of but one specimen of *Amblystoma punctatum* has been examined. This animal was taken from a pond in late March during the mating season. The lobules had presumably been emptied or partially emptied some time earlier, for regeneration was well under way. Many lobules were almost entirely filled with secondary spermatogonia. The interstitial cells had reached a late stage of development. They were, indeed, well on their way toward total disappearance around the apices of the lobules, and even where most numerous, near the periphery of the testis, an extremely large number of them had nuclei with the chromatolytic figures indicative of degeneration. They, as well as the degenerating portions of the lobule, contained numerous lipid droplets. Fuchsinophile granules were also present in large numbers. The cells were arranged in sheaths around lobules; these cut in cross-section gave the usual 'epitheloid ring' picture.

Testes of *Plethodon glutinosus*, *Plethodon cinerius erythronotus*, *Spelerpes bilineatus*, and *Gyrinophilus porphyriticus* have also been examined. All of these were taken prior to the formation of spermatozoa. No interstitial cells were present between the lobules of developing spermatogonia; neither were any present in an extensive shrunken caudal portion of the testis, such as is found in *Desmognathus* during the summer months. Interstitial cells, if formed in these animals, probably persist but a short time, as in *Diemyctylus*. Material for a further study of the testes of these forms is now being collected; this will be reported separately at some later date. That interstitial cells were not found in the specimens examined bears out the statement already made: that they show no constant, definite relation to the progressive phases of spermatogenesis, but are absent from regions in which developmental processes are in progress.

#### DISCUSSION

Throughout the preceding description the relation of interstitial cell development in urodeles to the condition within the lobule has been emphasized. This, briefly stated is: 1) that throughout their period of development, from their early mitoses

and beginning enlargement to their period of maximal development, they are associated with the degeneration of lobules which have completed a spermatogenetic cycle; 2) that their regressive changes, whether degeneration or return to a stromal cell type, unless occurring before the lobules of the region regenerate (as, for example, in *Desmognathus*), take place invariably if they become crowded between lobules of developing germ cells, or are crowded to the periphery of the testis by such growth.

Similar correlations between interstitial cells and the tubuli contorti have been described for mammals, atrophic changes in the tubules being accompanied by hypertrophy of the interstitial cells. Durck found hyperplasia and hypertrophy as accompaniments both of imperfect development and secondary atrophy of the tubules. Such, one would infer, is the usual condition in cryptorchidism; it is the normal occurrence, too, after vasectomy or treatment with Roentgen rays. Simmonds<sup>9</sup> finds that in case of the regeneration of the tubules after this latter treatment (which frequently fails to entirely destroy all of the germ cells) the hypertrophied condition of the Leydig's cells disappears. His inference was "that maintenance of the sex characters, by means of an internal secretion, is a function of both the spermatogenic cells and the cells of Leydig; that under normal conditions the cells of Leydig are few in number, but, after the destruction of the spermatogenic cells, there is vicarious intervention on their part, in consequence of which they undergo proliferation." It is, of course, interesting to note that in cryptorchid testes, such 'vicarious intervention,' according to Bouin and Ancel, may be insufficient, and the animals then are subnormal in secondary sex attributes, sexual instinct, or development of the genital tract. It may be questioned whether such interstitial cell 'intervention' can persist alone for any length of time after complete atrophy and disappearance of the tubules from the testis. The cells do not long persist in the urodeles after complete disappearance of the lobules they surround, but, according to Simmonds' interpretation, this would be because the germ cells

<sup>9</sup> Quoted by Biedl, page 397.

of the next cycle are by that time sufficiently developed so that interstitial cell 'intervention' is not required. What might occur if all germ cells in the urodele were destroyed is, of course, problematic. In cases of extreme atrophy of the testis there is a more or less complete disappearance of the interstitial cells (Cushing's Case XXXII, p. 277, and Biedl, p. 396).

The work of Steinach, who claims to have 'restored' old male rats to youthful vigor and activity, is suggestive in this connection, as the stimulation was induced by causing testicular degeneration. Steinach ligatured the testis of one side, leaving the other intact. The ligatured testis of the 'restored' animal was found to contain greatly developed interstitial cells, while its germinal tissue had atrophied. The period of persistence of the 'restored' condition was not stated, the animals presumably having been sacrificed before a return of senility.

The association of the interstitial cells with degenerative changes, in the urodele testis, is I think, clearly demonstrated, both by my own observations and those of Champy. The significance of such an association is another question. Though the absence of typical interstitial cells from regions where spermatogenesis is going on precludes the possibility of their serving the 'trophic' function sometimes ascribed to them, the stromal cell, from which they develop, may serve some such purpose. As these cells always surround the lobule in such a way as to be between it and the blood vessels, the nutriment reaching the germ cells must either pass through their cytoplasm or traverse intercellular spaces adjacent to it. Conversely, if products formed within the lobule are absorbed through blood or lymph channels, such products must first pass through or between these stromal cells. These might, therefore, be expected to react (as they do) to changes in the metabolic condition of the lobule.

What, then, is the source of the products (granules, lipoids) seen when these stromal cells begin hypertrophy? Are they derived from substances from within the lobule or are the materials forming them taken from the flow of nutriment passing from blood vessel to lobule? To my mind, both sources seem possible and probable. The hypertrophied cells doubtless receive end

products of intralobular degeneration; their position around the lobule, rather than around the blood vessels, suggests this. There is no evidence that the products of degeneration find their way out of the testis via the collecting duct and ductuli efferentes. Their return to the circulatory system by way of the stromal cells seems probable, with the consequent assumption by the latter of the interstitial-cell character. The disappearance of the interstitial cells shortly after the degeneration of the lobule is completed is further indication of their dependence upon its influence. On the other hand, the hypertrophy of stromal cells around the lobule may be in part due to their accumulation of substances normally passed on into the lobule, but now piled up just short of their former destination through the inability of the Sertoli cells to utilize the accustomed quantity. This, presumably, is the explanation of the occasional presence of lipoids in the stromal cells during the progressive phases of spermatogenesis, when degeneration within the lobule is relatively improbable. Supply and consumption are so balanced, as a rule, during these phases that there is little or no lipoid demonstrable in the stromal cells. Consumption being curtailed, lipoids accumulate.

It is suggested that the accumulation of lipoids within the lobule is, similarly, an expression of the acquisition by the Sertoli cells of materials which they can no longer utilize. A comparatively enormous amount of lipoid is seen in the lobule, in *Necturus* especially, before the interstitial cells reach their maximum. That the diminished ability of the Sertoli cells to receive materials, after the germ cells which utilize them have left the lobule as spermatozoa, should result in a secondary 'backing up' of the incoming supply into the stromal or interstitial cells, seems within the realm of possibility. There is the further possibility that by their accommodating hypertrophy at this time these cells may help preserve the nutritive balance for the region in which they occur, and, by so doing, prevent disturbance of the normal growth processes of the testis.

The interstitial cells of urodeles may also serve to avert a too sudden collapse of the greatly distended lobules, thus preventing

interference with the normal circulation through the interlobular vessels. In other animals (e.g., woodchuck, mole, corvus) in which there is a marked change in the volume of the testis following spermatogenesis, the reduction in size of the tubules is accompanied by such growth of interstitial cells as to prevent complete collapse of the organ. In the mammalian ovary, similarly, the development of interstitial cells around atretic follicles and the hypertrophy of follicular and stromal elements during the formation of the corpus luteum may meet a mechanical need, even though the existence of such a need is not of itself sufficient to initiate the growth processes mentioned. Chemical as well as mechanical factors are involved, and the impossibility of separating the two stands in the way of any determination of their relative importance.

Biedl says: "If we summarize all that is known concerning the internal secretory tissue of the male genital gland, we are led to the inevitable conclusion that the hormone which gives to the organism its male characteristics, is elaborated in the cells of Leydig in the interstitial tissue. . . . We are justified in describing them in their totality as an interstitial gland."

The writer does not wish to be understood as asserting the inapplicability of this statement to urodeles. Other structures of the vertebrate body having, in their original state, as little adaptation for glandular activity, develop into endocrine organs of more or less recognized status. Hence, despite the conditions which invariably call the interstitial cells of urodeles into being, there is yet the possibility that their lipoids or degeneration products, after entering the circulation, are capable of influencing the organism in specific ways, either alone or in combination with other testicular elements. It seems desirable, however, to offer a few brief comments on the relations of the interstitial cells to which one might look for confirmation of their supposed secretory function in the urodeles.

Morphologically, they do not possess the usual relations of an endocrine gland with respect to blood vessels. They develop primarily around the lobule; any relation to the blood vessel is secondary and incidental—i.e., through crowding. Though

the cells possess noticeable polarity (nucleus toward one end, enlarged centrosphere, granules, etc., toward the other, in *Necturus*), they show no particular orientation of the cell body with respect to either lobule or blood vessel.

The inconstancy of the interstitial cells in urodeles would further tend to preclude their recognition as a gland in the morphologic sense of the term. Lacking for several weeks in *Necturus* and *Diemyctylus*, and probably for several months in *Gyrinophilus*, *Plethodon*, and *Spelerpes*, they persist in *Desmognathus* until those of the succeeding year develop, and hence in the mature male are, as a rule, never absent. In any event, in the animals in which they appear, the individual cell is of transitory character—a mere temporary modification of a stromal cell. The absence of the cells from the sexually immature animals I have studied is further evidence of their lack of individuality as a cell type.

If the cells subserve the purpose of elaborating an internal secretion of specific action, that is, if they constitute a gland in the physiologic sense, their absence from the sexually immature, and temporary absences from the adult, seem anomalous, to say the least.

A constant relation of their development to phases of the sex cycle (other than to lobule degeneration, in which relation all urodeles agree) seems difficult to ascertain, as in many urodeles so little of the life-history and mating habits is known. It would appear that in *Necturus* and *Cryptobranchus*, and perhaps in *Diemyctylus* as well, the late summer or fall matings occur at a time when interstitial cells have been absent for several weeks, and too soon after the emptying of the lobules for the new interstitial cells to have reached any conceivable state of secretory activity. A spring mating in *Necturus*, if it should occur, would take place when the cells were at or somewhat past their maximum; the spring mating of the one *Amblystoma* male I have examined must have occurred toward the later part of their cycle. That interstitial cells in each of these cases regulate the appearance of secondary sex characters, as well as the instincts of the mating season, can hardly be given credence.

The absence of these cells from the sexually immature animals I have examined prevents faith in the potency of their influence on development of the secondary sex characters, male sexual organs, etc. If their appearance is delayed until after the first extrusion of spermatozoa, one might well question their influence upon any of the phenomena of sexual maturity. The number of sexually immature animals I have examined is so small, however, that the possibility of an interstitial cell proliferation at some other stage of development has not been absolutely excluded, though conditions under which interstitial cells appear in the adult render such a proliferation in the sexually immature animal improbable.

That the cells act to regulate growth processes in the germ cells seems improbable. The phenomena of maturation, for example, would occur in *Necturus*, *Cryptobranchus*, and *Gyrinocheilus* during their absence. That their disappearance from the testis removed an inhibitory influence, thus permitting maturation to proceed, is also improbable, since in *Desmognathus* they are yet numerous through the entire period in which this occurs, though of course isolated in the caudal shrunken portion of the testis. Champy ('13) inclines strongly to the belief that the regression of the interstitial cells, i. e., the resorption of their 'secretion' products, coincides with the onset of spermatogenesis.<sup>10</sup> The persistence of the interstitial cells throughout the entire year in *Desmognathus* indicates that spermatogenesis may be initiated without the resorption of these elements. In the forms Champy has examined, the spermatozoa are matured in autumn, but retained in the testis until the mating period in the following spring; the interstitial cells, therefore, develop only in the spring and undergo regression immediately preceding the onset of spermatogenesis. In *Diemyctylus*, which has a fall as well as a spring mating, numerous lobules are emptied in autumn and interstitial cells develop around them. These apparently undergo regression within a short time after their formation, exactly as they do when developed in the spring.

<sup>10</sup> Champy, in his graphs, fixes the curve of spermatogenesis by the number of spermatocytes I in prophase.

Spermatogenesis, however, is not initiated by this autumnal development and regression.

Similarly, it is not apparent that the development of the interstitial cells is responsible for the checking of the spermatogenetic processes toward the close of the cycle. In those urodeles with a slow spermatogenetic 'wave' (*Desmognathus*, *Diemyctylus*) a 'boundary plane' is established early in the season through the degeneration of the germ cells in lobules near the anterior end of the testis; only the germ cells caudal to this 'boundary plane' then mature as spermatozoa during the current season. The 'boundary plane' is established, however, before any lobules are emptied, and hence its appearance does not coincide with any proliferation of interstitial cells. Neither is there any marked change at this time in those interstitial cells of *Desmognathus* which were formed at the close of the preceding cycle; in *Diemyctylus* such cells have already disappeared from the testis.

On the whole, therefore, this study of the Leydig's cells in urodeles adds little or no weight to the evidence in favor of their interpretation as an endocrine organ. *It does, however establish—and more clearly than can be determined in the anuran, sauropsid, or mammalian testis—the close relation of the cells to regressive and degenerative changes in the lobules.* The part, if any, these cells may play in the organism, during their temporary hypertrophy or transformation from stromal cells, remains more or less problematic.

#### SUMMARY

1. In higher vertebrates the interstitial cells of the testis have been found to vary greatly in their correlations with the phases of the spermatogenetic cycle, though as a rule their greatest development appears to be correlated with the later or regressive phases.

2. Such variations as appear are believed to result from the complexity of the testis in these higher forms rather than from intrinsic differences in the cells themselves in the different animals investigated.



3. The testes of urodeles are more favorable for the determination of interstitial cell correlations.

a. In the individual lobule the germ cells are all at the same stage of development at any one time, or approximately so.

b. The caudocephalic movement of a 'spermatogenetic wave' gives a succession of developmental stages in a single testis, each stage localized in a region in which the germ cells are all similarly developed.

c. At the close of the cycle the spermatozoa are extruded and only the Sertoli cells remain in the lobule; these soon degenerate.

d. The lobules are regenerated from residual spermatogonia persisting only in their apices; degenerating and regenerating portions of the lobule are therefore distinct.

e. Regeneration is so long delayed in some urodeles that it begins only after the degeneration of the emptied lobule has been completely effected; this results in an even more complete separation of the progressive and regressive phases of spermatogenesis.

4. The development of the interstitial cells in urodeles is correlated with the regressive phases of the spermatogenetic cycle.

a. During the initial stages of spermatogenesis only flattened or spindle-shaped cells of the connective-tissue-cell type appear.

b. These cells may increase in number by mitotic division, this occurring usually before the spermatozoa have completely left the lobule.

c. Following the extrusion of the spermatozoa, these cells round out, increase greatly in bulk, accumulate lipoids, and become typical interstitial cells.

d. Such interstitial cells in any urodele have been found to develop chiefly in association with lobules undergoing degeneration at the close of the spermatogenetic cycle.

5. The urodele interstitial cell is but a temporary modification of a stromal cell.

a. Interstitial cells developed around an emptied lobule disappear when the degeneration of the lobule is effected or soon thereafter. Though many of them degenerate, others clearly revert to the original stromal-cell type.

b. The absence of interstitial cells in the sexually immature male further precludes their recognition as a distinct cell type.

c. These cells cannot be considered as constituting a gland in the morphologic sense of the term.

6. There has been found no evidence that the interstitial cells constitute a gland of internal secretion.

a. Secretion products other than lipid substances have not been observed.

b. Interstitial-cell development bears no constant relation to the mating period and its phenomena.

c. The maturation phenomena appear in no way dependent upon the presence of interstitial cells; neither do they always immediately follow the disappearance of these elements.

7. The value of the interstitial cells to the organism during their period of hypertrophy remains problematic.

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## PLATE 1

## EXPLANATION OF FIGURES

8 Testis of *Necturus* July 31st; cephalic portion tangential section, cutting the lobules in cross-section. Shows the distention of the lobules characteristic of this period. The lobules are filled with spermatocytes I. Bouin's fluid; 8 $\mu$ ; copper hematoxylin.  $\times 120$ .

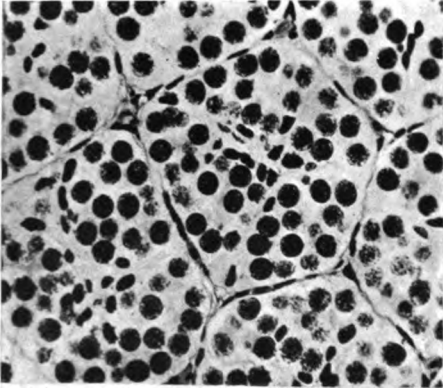
9 Portion of above section under higher magnification, to show the interlobular nuclei of elongated form.  $\times 300$ .

10 Testis of *Necturus* October 7th; caudal region. Lobules cut in cross-section. Compare with figure 8 as to shape of the lobules, from which now the mature spermatozoa have been partially extruded. Groups of spermatozoa within the lobules are deeply stained. A mitotic figure is seen in the interstitial-cell sheath, near the upper edge of the photograph. Zenker's fixation; 6 $\mu$ ; iron hematoxylin.  $\times 154$ .

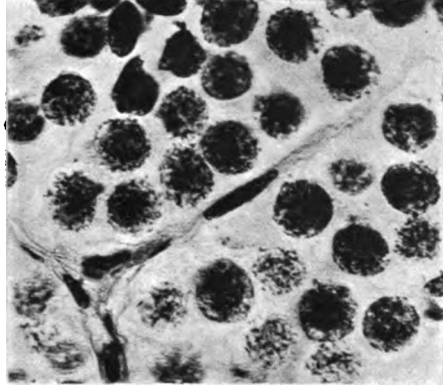
11 Same section as in figure 10, but showing the lobules cut in longitudinal section. Interlobular tissue greatly increased in prominence; nuclei less flattened than in figures 8 and 9; mitotic figures abundant (one appears near bottom of photograph).  $\times 120$ .

12 Testis of *Necturus* October 30th; tangential section of caudal region. The lobules, which are here cut in cross-section, contain only degenerating Sertoli cells and are surrounded by prominent epithelioid rings of interstitial cells. Regaud's fixation; copper hematoxylin; 5 $\mu$ .  $\times 154$ .

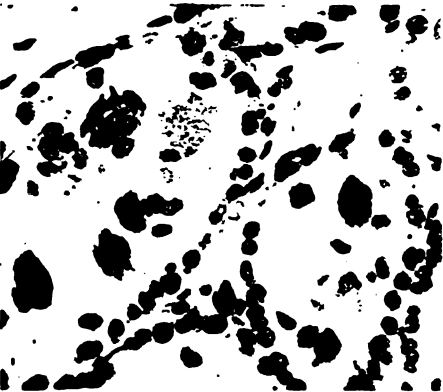
13 Testis of *Necturus* April 8th; extreme anterior portion. Shows considerable lipid, blackened by osmic acid, within the degenerating lobules, but only a few scattered interstitial cells (as at *x*). Bensley's fixation; 5 $\mu$ ; no stain.  $\times 120$ .



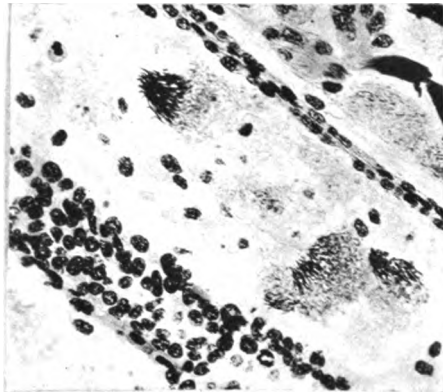
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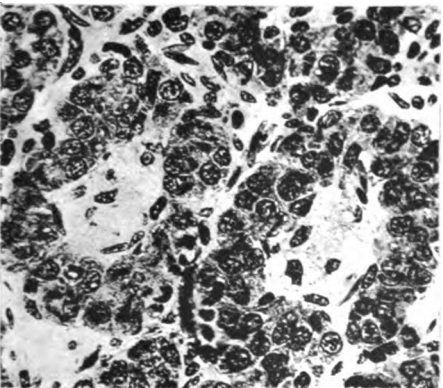
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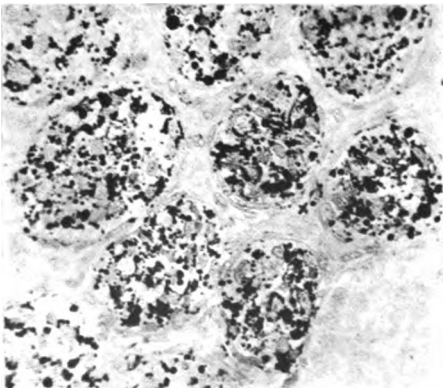
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## PLATE 2

### EXPLANATION OF FIGURES

14 Testis of *Necturus* April 28th; transection through the middle portion. Regenerating lobules contain secondary spermatogonia. The lipid in the degenerating lobules is blackened by osmic acid. Interstitial cells with a comparatively small lipid content occupy the spaces between these lobules. This section is comparable to one through the caudal portion of the testis in the fall or early winter. Hermann's fixation; 8 $\mu$ ; no stain.  $\times 36$ .

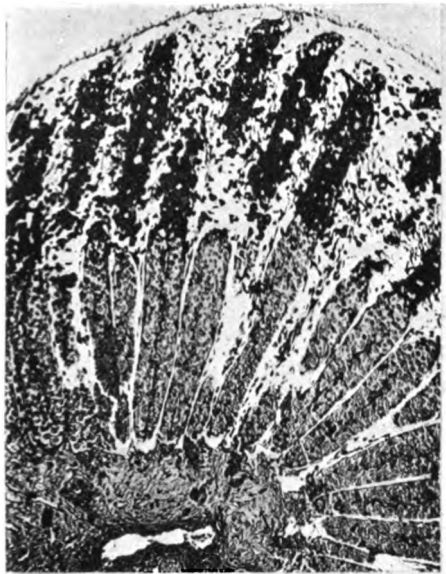
15 Testis of *Necturus* June 9th; caudal portion of the organ. The photograph includes the distal ends of two regenerating lobules, between which are compressed many interstitial cells, some of which will doubtless revert to the original stromal-cell type. At the end of the lobule at the right is a bit of a degenerating lobule, now greatly reduced in size, but containing considerable lipid. Bensley's fixation; 6 $\mu$ ; no stain.  $\times 120$ .

16 Same as figure 15, but with the lobules cut in cross-section through their distal ends; interstitial cells are here crowded between lobules instead of being arranged in distinct rings as during their developmental stages illustrated in figures 10 and 12. At the left is a region into which the tips of the developing lobules have not yet extended. The large fat droplets here are in a degenerating lobule.  $\times 120$ .

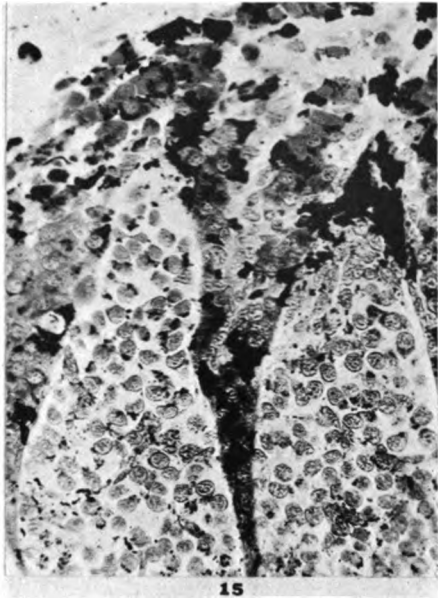
17 Testis of *Necturus* July 21st. Median sagittal section. A few hypertrophied, fat-laden interstitial cells are scattered along the periphery of the testis (right). The fat droplets in the center are in the Sertoli cells of the lobule. Only very rarely are any interstitial cells found between lobules at this time. Flemming's fixation; 5 $\mu$ ; no stain.  $\times 120$ .

18 Testis of *Necturus* April 3rd; caudal portion. Shows the massing of interstitial cells near the periphery of the organ; degenerated lobules and blood vessels are crowded between masses and cords of these cells. A group of degenerating nuclei appears in the upper left of the photograph. Giant centrospheres, now at their maximal size, are seen as lightly stained areas associated with the normal nuclei. Allen's fluid; 10 $\mu$ ; iron hematoxylin.  $\times 154$ .

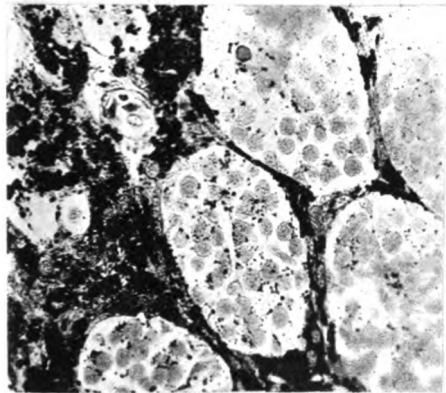
19 A portion of the same section shown in figure 18, but under higher magnification ( $\times 300$ ). A blood corpuscle in the upper left corner of the photograph indicates the position of a blood vessel; the interstitial cells, through crowding, may become intimately associated with blood vessels at this time, although not so associated in the earlier part of their cycle.



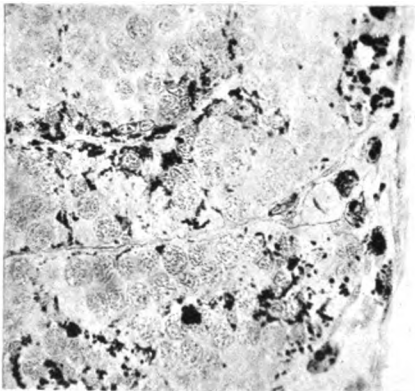
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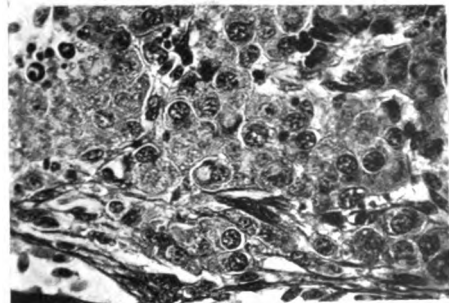
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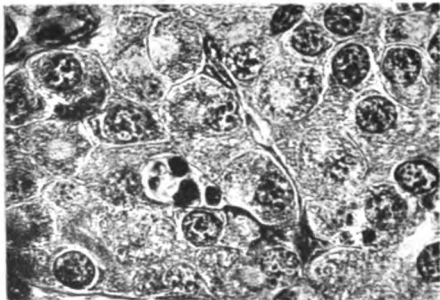
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### PLATE 3

#### EXPLANATION OF FIGURES

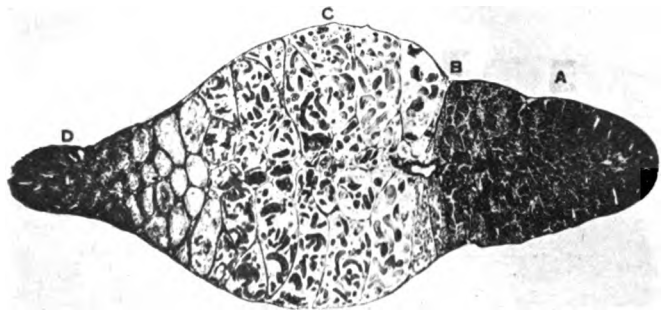
20 Testis of *Desmognathus*. Longitudinal section of a single enlargement to show the condition in early autumn, after some of the lobules have been emptied. *A*, region occupied by spermatogonia; *B*, boundary plane; *C*, lobules yet filled with spermatozoa; *D*, empty lobules surrounded by various stages in the development of interstitial cells.  $\times 16$ .

21 Testis of *Desmognathus*. Caudal portion of the section shown in figure 20, under higher magnification ( $\times 43$ ). Stages in the enlargement of the stromal cells are readily seen; typical interstitial cells are present in the caudal region first emptied. Note the condition of the interlobular tissue while spermatozoa are present in the lobule as compared with that following their extrusion.

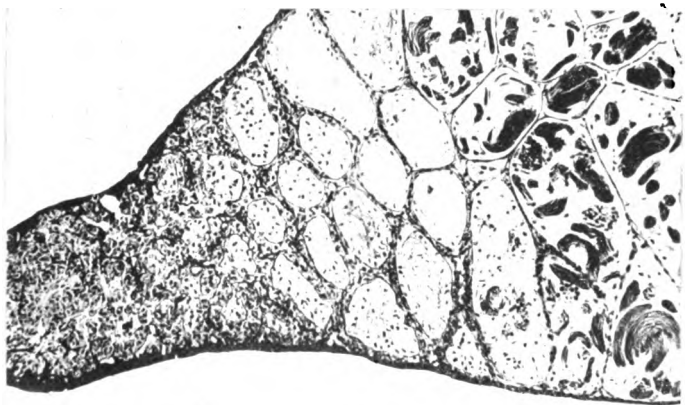
22 Interstitial cells of *Desmognathus* at their maximal enlargement, during the summer months. An area of dense cytoplasm containing the idiozome appears at one side of the nucleus as in the interstitial cells of *Necturus*. The smaller granules and mitochondria are largely grouped around this idiozome; the peripheral cytoplasm of the cell is left vacuolated through the solution of larger lipid droplets. Flemming's fixation; iron hematoxylin.  $\times 600$ .

23. Testis of *Salamandra atra*; lobules are yet partially filled with spermatozoa, but the interlobular nuclei are rounding out and increasing in numbers. At the lower left is a mitotic figure, bulging somewhat into the adjacent lobule. Zenker's fixation;  $10\mu$ ; iron hematoxylin.  $\times 120$ .

24 Testis of *Salamandra atra*; section of a region caudal to that shown in figure 23. The interstitial cells here form prominent rings around the degenerating lobules. Their osmicated lipoids make them especially prominent structures in this preparation. No stain.  $\times 120$ .



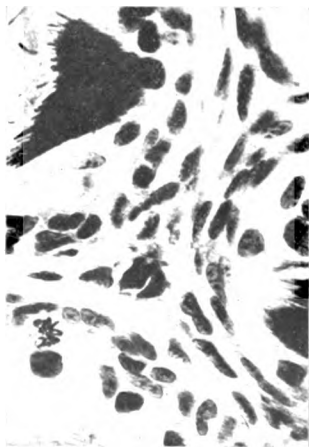
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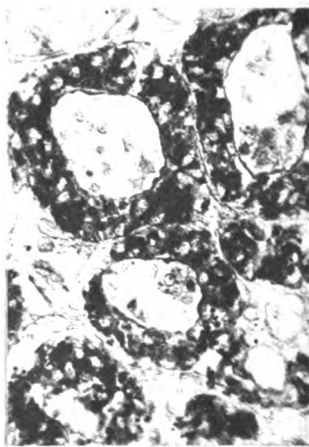
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## PLATE 4

### EXPLANATION OF FIGURES

Interstitial cell types from the testis of *Necturus*. All figures in this plate were outlined with the camera lucida; magnification, 750 diameters. All are taken from preparations fixed in Bensley's fluid (osmic-dichromate-acetic) and stained with acid fuchsin and methyl green. Lipoid droplets appear black, mitochondria and fuchsinophile granules red, nuclei green.

25 Stromal-cell type seen during the summer months between lobules of spermatogonia or spermatocytes. The nuclei may be even more flattened, as is shown in figures 8 and 9.

26 Cells surrounding partially emptied lobules in the caudal end of the testis in early October. Numerous mitochondria have now appeared; no cell boundaries are as yet distinguishable. At the upper right is a connective-tissue cell which has not begun transformation to an interstitial-cell type.

27 One of the mitotic figures so numerous in early October among developing interstitial cells. Among the red granules are a few of larger size than those shown in figure 26.

28 Cell in a more advanced stage of development than are those shown in figures 26 and 27. The nucleus is more rounded, cell boundaries are distinct and many fuchsinophile granules are decidedly larger than the granules (mitochondria?) in figure 26.

29 Cell from the caudal end of the testis in late October. Lipoids blackening with osmic acid have appeared, many of them of the same size as the fuchsinophile granules. Nuclei are now approximately spherical and cell boundaries are distinct.

30 Cell from the same section as figure 29, but showing the idiozome, surrounded by numerous fuchsinophile granules and blackened lipoid droplets.

31 Cell from the caudal portion of the testis in April, showing giant centrosphere with idiozome and centrioles. Interstitial cells of this type predominate in the early spring.

32 Degenerating cell lying adjacent to the cell shown in figure 31. Cells of this type are comparatively few in number in the earlier part of the spring.

33 Degenerating cell (or cell mass?) common in the testis in May. The bodies at the right are apparently masses of cytoplasm containing small fuchsinophile granules (mitochondria?). It is suggested that these may give rise to such solidly staining fuchsinophile masses as the one at the left.

34 to 39 Cell types seen in the testis in June and July. Occasionally cells of the type of 35 or 36 may persist through the summer, but in the majority of the animals examined their disappearance is complete. Figures 38 and 39 show cells of a type frequently encountered at the periphery of the testis at the time the larger cells are becoming few in number.

R. R. HUMPHREY

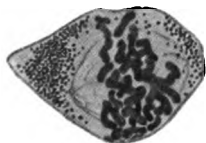
D.D. ADAMS, Jr.



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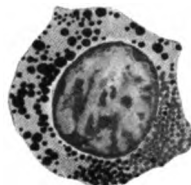
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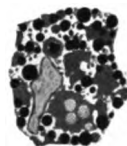
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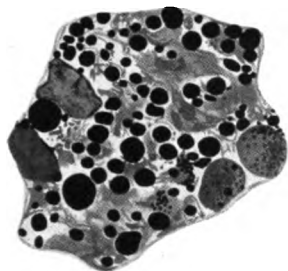
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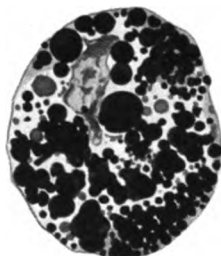
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Resumen por la autora, Ruth Stocking Lynch.

El cultivo in vitro de las células del hígado del embrión de pollo.

Los mejores cultivos de células del hígado en la solución de Locke-Lewis se obtienen utilizando embriones desde los 5 a los 12 días de la incubación. Las células emigran sin dividirse a lo largo del cubreobjetos, desde el trozo depositado en el cultivo, formando una lámina o membrana. Las mitocondrias aparecen en abundancia bajo forma de gránulos más o menos irregulares de tamaño variable. En algunas ocasiones puede observarse la presencia de gránulos de bilis de color verde brillante. Dichos gránulos presentan una marcada afinidad hacia el rojo neutro. En las células se acumulan gradualmente gránulos citoplásmicos o de degeneración, así como vacuolas; se tiñen rápidamente con el rojo neutro y más despacio con el azul trypan, y cuando mueren pierden rápidamente el rojo neutro, conservando el azul durante mayor tiempo. Cuando se emplean simultáneamente ambos colorantes cada gránulo fija ambos variando su coloración (roja, violeta, lavándula, azul) según la cantidad relativa de ambos colorantes. Los glóbulos de grasa son más numerosos en las células de los embriones más viejos, pero no aumentan con la edad del cultivo. La degeneración tiene lugar mediante vacuolización o formación de ampollas. El mesenquima (probablemente endotelio) aparece a menudo bajo la forma de un retículo laxo en los cultivos. Células emigrantes aisladas (clasmatocites?) son relativamente escasas. Las membranas mesoteliales se observan muy raras veces. En el medio de cultivo se acumula gradualmente fibrina o un producto semejante a dicha sustancia, procedente del trozo cultivado, la cual a veces oscurece más o menos el crecimiento.

Translation by José F. Nonidez  
Cornell Medical College, New York

## THE CULTIVATION IN VITRO OF LIVER CELLS FROM THE CHICK EMBRYO

RUTH STOCKING LYNCH

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TWENTY-FIVE FIGURES

### INTRODUCTION

The liver of the chick embryo is composed of at least two different types of cells, the parenchyma or liver cells proper and the endothelial cells of the sinusoids [Minot<sup>1</sup>]. In addition, much of the surface is covered by the mesothelial cells of the peritoneum. According to Minot, after 96 hours of incubation the embryonic chick liver contains no mesenchyme. In our cultures four types of cells were found growing out from the explants: liver cells, endothelium, mesothelial cells, and wandering cells. The liver cells migrated out from the explant as a membrane or sheet of cells somewhat similar to the membranes formed by the endodermal cells from cultures of the intestine though less extensive. The growth of the endothelium was more or less reticular.

In some of the successful cultures only liver cells proper and wandering cells were present (fig. 1), but in most of them endothelium was also found (figs. 19, 20, 23). A few cultures showed mesothelial cells as well. The endothelium grew either in close association with the liver cells or more or less isolated from them. In many cultures the liver cells overgrew a reticulum of endothelium which lay next to the cover-glass (fig. 20). In others there was complete separation between the two types of cells, endothelium only growing out from one part of the ex-

<sup>1</sup> Minot, C. S. 1900 On a hitherto unrecognized form of blood circulation without capillaries in organs of vertebrata. *Proc. Boston Soc. of Nat. Hist.*, vol. 30.

plant, or from one explant when more than one was present, liver cells only growing out from another part of the same explant or from another explant. Wandering cells occurred anywhere in the cultures, on or among the other cells or entirely isolated from them. They were much more abundant in cultures showing little or no growth than in those actively growing.

According to Beck<sup>\*</sup> clasmatoocytes are rare in the liver of a seven-day chick embryo, and the wandering cells which appeared in our cultures seem to differ somewhat from them.

#### METHOD

Explants were made from embryos of five to eighteen days' incubation. These were examined at all stages of cultivation from the time of planting to the death of the culture. The culture medium used, Locke-Lewis solution (Locke's solution plus 0.5 per cent dextrose plus 10 to 20 per cent chicken bouillon), was the same throughout the entire series of experiments.

The living cultures were studied with and without the use of the various vital dyes. Janus green, neutral red, and trypan blue were used. Cultures at various stages of growth were fixed in osmic acid vapor, in Zenker without acetic acid, in Zenker-formol, in Schaudinn, Bouin, and in iodine vapor. The first two fixatives were the most successful. Preparations were stained in hemateine and hematoxylin alone and in combination with eosin, carmine and methyl green; in carmine, iodine, crystal violet and Gram's stain; in safranin and licht grün, and in acid fuchsin and methyl green. The results obtained with carmine, iodine or crystal violet were unsatisfactory. Neither of the two glycogen stains, carmine and iodine, gave definite staining of the granules; with each of them the nucleus, nucleolus, and debris stained deeply and the cytoplasm lightly.

All drawings were made with the camera lucida, from the living cell. The photographs are of the fixed preparations.

<sup>\*</sup> Beck, C. S. 1919 The relative distribution of clasmatoocytes in the various organs of the seven-day chick embryo. *Anat. Rec.*, vol. 16, p. 143.

## LIVER CELLS

*Migration*

The time at which migrating cells first appeared varied greatly. In some instances the first cells appeared within 4 hours after planting; in most cases within 24 hours; in a few not before 48 or more hours had elapsed. One culture, which eventually gave rise to an extensive growth, had only a few migrating cells 72 hours after planting. There appeared to be some correlation between the age of the embryo and the beginning of growth, the explants from younger embryos tending to show migrating cells earlier than explants from embryos of nine or more days' incubation. Trypan blue cultures also were characterized by early and rapid migration of liver cells. These cultures gave extensive growth much more quickly than cultures grown in media without the dye, one trypan blue culture reached the climax of its growth within 18 hours after planting. In most cultures the climax occurred 48 to 72 hours after planting; in some cases, however the best growths appeared much later, even as late as 144 hours.

The best growth of liver cells obtained was  $27\mu$  wide and occurred 120 hours after planting. It was one of a set of ten cultures taken from an embryo of eight days' incubation, which gave the best growth results in the entire series of experiments. Fresh bouillon was used in making up the Locke-Lewis solution for this set. Of these ten cultures, eight showed extensive liver-cell growth within 24 hours after planting; one showed extensive growth only after 48 hours had elapsed; the tenth showed only endothelium. Of the eight which showed wide growth within 24 hours, one was infected while staining with neutral red and had to be discarded, and one did not grow any further. Of the other six, one began to degenerate on the fifth day, four on the sixth day, and the sixth was in fine condition on the sixth day when it was fixed (figs. 19, 20). The one which did not show good growth until 48 hours after planting did not begin to degenerate until the seventh day. In many cultures degeneration began at 72 hours; in others the cells were in good condition after



four days of cultivation. In the majority death had occurred by the sixth day; in some not until the ninth day (figs. 1a to 1f).

### *Character of growth*

In their manner of growth, and to some extent in character, the liver cells resemble endodermal cells from explants of the intestine, although they grow out from the explant much less readily and much less rapidly. The first liver cells appeared at the edge of the explant in one of three ways: as individual projecting cells, as a small triangular sheet of cells, or as a long narrow sheet along the margin of the explant (figs. 1, 3, 4). The second arrangement was the most common. The cells shifted somewhat and changed shape, drew back into the explant, or extended farther out (figs. 3, 4). In many cases individual cells or groups of cells became entirely isolated from the rest of the growth, and would creep out some distance from it (figs. 1e, 24, 25). These were usually greatly flattened and therefore afforded an excellent opportunity to observe behavior and details of structure.

In the best growths, before degeneration set in, the membrane had a fairly regular contour with a finely serrated edge (figs. 3, 4, 19). When degeneration began this edge became ragged, holes formed in the body of the membrane, and the whole sheet began to break up (figs. 1d, 1e). In a few cultures this irregularity of contour was present from the beginning (figs. 1a, 1b). Many growths obtained from explants made in the culture medium plus trypan blue, were ragged and had numerous isolated cells.

### *Relation of migration to other factors*

*Age of embryo.* Growth was obtained from explants of five to sixteen day embryos, good growths from explants of five to twelve day embryos. No migrating liver cells were obtained from explants of embryos older than fifteen days. Seventy-three series with a total of 450 cultures, were made from embryos of five to twelve days' incubation. Of these 250 (55 per cent) showed migration of liver cells and 91 (20 per cent) gave good liver-cell growth. Nine series with 33 cultures were made from

embryos of thirteen to sixteen days' incubation. Of these 23 (70 per cent) gave some liver-cell growth, but in none was it very extensive. Two series with a total of 12 cultures were made from seventeen to eighteen day embryos. In these no liver-cell growth was obtained.

*Size of explant.* The length and width of fifty-one explants were measured and their areas roughly computed. The explants varied in area from approximately 300 to 12,000 square  $\mu$ . No correlation was found between the area of the explant and successful growth; good growths occurred from explants of all sizes.

#### *Cell division*

Although endothelial cells divided actively in many of the cultures, no division of liver cells was observed. In the living cultures and on the fixed slides they frequently showed elongated nuclei and nucleoli, and one amitotic division of the nucleus without subsequent division of the cytoplasm was observed in a living culture. Many liver cells of the living cultures and of the fixed preparations contained two and sometimes three or four nuclei.

#### *General cell characters*

The liver cells in that part of the growth membrane near the explant were thick, polygonal, and opaque, having a greenish-gray color due to the dense packing of their cytoplasm with mitochondria. The marginal cells and the cells entirely isolated from the explant were flattened and much clearer than the other liver cells, but were never as clear as the endothelium. Their processes, although quite short, were usually free of mitochondria as were also the free edges of the cell (figs. 2, 8, 9, 10, 12, 22, 24, 25). In most of the cells ectoplasm and endoplasm could be distinguished. In the endoplasm were seen the nucleus, the mitochondria, bile granules, neutral red granules, and fat globules, all closely packed together and showing only slight shifting movements. In the ectoplasm there were usually some neutral red granules, and here they were very active, even large ones moving about to a considerable degree.

The nucleus was spherical and clear with a very distinct boundary which made it conspicuous under all magnifications. In the isolated cells and in the compressed cells of the interior of the growth membrane the nucleus was usually central, while in the marginal cells it was more proximal, with the greater mass of the cytoplasm lying between it and the free edge of the cell (figs. 3, 4, 21, 22, 23). As has been stated, cells with two or more nuclei occurred quite frequently (figs. 6, 12). The nucleolus was usually single and its location varied greatly. In some cases it was even in contact with the nuclear boundary. Sometimes two or more nucleoli were present (figs. 6, 13, 14, 17, 18, 22). The nucleolus of the liver cell was shorter and stouter than that of the endothelial cells and often had a granular appearance.

#### *Mitochondria*

The cytoplasm of the thick compressed cells of the interior of the growth membrane was packed with mitochondria, the outlines of the individual granules being for the most part indistinguishable. In the cells at the margin of the growth and in the isolated cells the clear ectoplasm of the processes and of the free cell edge was easily distinguishable and did not appear to contain mitochondria, while the endoplasm was full of them. They were somewhat scattered, especially in the flattest cells (figs. 5, 6, 7, 8, 9, 10, 12, 13, 17, 24, 25). They were not constant in shape, size, position or number. In some cells most of the larger mitochondria occurred near the periphery of the endoplasm, but large granules were often found in contact with the nucleus or scattered in the endoplasm.

The mitochondria varied in length from 1.5 to 4  $\mu$ . and in width from 0.5 to 1.5  $\mu$ . In shape they were spherical, oblong, triangular, pear-shaped or entirely irregular. Large blocks sometimes occurred. In some cultures they were quite long and bent (figs. 6, 8, 21), but were never thread-like as in the endothelium. The smallest granules were often arranged linearly, and when not quite in focus had the appearance of a long knotted thread. The mitochondria usually varied in size and shape in a single cell (fig. 21); but on the other hand, all the mitochondria in a cell, or

even in all the cells of a culture, were sometimes much alike in size and form (figs. 22, 24).

No correlation between size, shape or number of mitochondria and any other factor was found. All variations occurred in cultures of all ages, from embryos of all stages of incubation. Cells lying side by side and having, as far as possible, the same history and the same environment sometimes showed wide differences in character and amount of mitochondrial content (figs. 6, 8).

Some of the mitochondria seemed to be quite fluid, as they bulged first in one place and then in another. The longer ones alternately bent and straightened and all moved about to a considerable extent. In many cells, especially flattened ones, individual mitochondria could be followed for some time and their successive changes in form and position observed. When vacuoles developed the mitochondria lay in strands of cytoplasm around them (figs. 22, 25). As the cells degenerated and died the mitochondria lost their clear-cut outlines, becoming swollen, shadowy, indistinct bodies of irregular spherical form, filling the whole cell and giving it the appearance of a mass of grayish granules (figs. 18c, d, e). If they had been previously stained with janus green they lost their color during this process.

The living mitochondria stained a uniform, deep greenish-blue with janus green; they did not stain satisfactorily with janus black no. 2. Only two fixatives—Zenker without acetic acid and osmic acid vapor—gave entirely satisfactory results for the mitochondria. The stains which gave good results after Zenker were haematoxylin and hemateine alone, hemateine and carmine, and Bensley's stain (figs. 21, 22). After osmic acid vapor, safranin, licht grün and hemateine gave excellent results (fig. 24). The successfully fixed and stained preparations include cultures from twenty hours to eight days old, from embryos of six to ten days' incubation.

The picture presented by the fixed preparations (figs. 21, 22, 24, 25) is quite similar in all essentials to that of the living cell, unstained or stained with janus green. The cytoplasm of the fixed compressed cell is packed full of mitochondria, their out-

lines being more or less lost in their crowding. In the flattened cells the mitochondria appear separated and even somewhat scattered, and are distributed throughout the endoplasm without apparent plan, differing markedly in size and shape even within the same cell. They all stained alike, however, with each stain, just as in the living cell they all took up the janus green in a uniform manner.

### *Bile granules*

In the liver cells and wandering cells of these cultures there sometimes occurred bright green masses of irregular form, usually about the size of the nucleolus or a little larger. These were probably bile granules. They were observed in cultures of two, three, four, and five days' cultivation, from chick embryos of seven, eight, and eleven days' incubation. The number of liver cells showing them was always few; often only two or three in an entire growth membrane. Wandering cells containing them were much more numerous; in one culture made from an eleven day embryo all the wandering cells showed large, bright green masses on the second day of cultivation. It is probable that these macrophagic cells took up these bile granules from the broken down liver cells of the explant.

The bile granules were evidently situated in the endoplasm of the liver cells; they exhibited no movement and usually lay close to the nucleus. They were never observed near the cell periphery. No staining of these granules with trypan blue was observed; in trypan blue cultures, cells containing both blue and green granules were found. With neutral red, however, they stained very deeply, more deeply than any other cell inclusion observed. When a dilute solution of neutral red was added to an unstained or a trypan blue culture the green granules changed to orange, then to brown, to reddish-brown, and finally to a very dark red. In cultures grown in a neutral red medium no green granules were to be found. When neutral red cultures were treated with potassium permanganate these masses gradually lost their red color until after a time, varying from a half to one and a half hours, they became a dark brown or even a brownish-

green. In one case most of the mass faded to a dark green except one small spot which remained red and another small spot brown. When trypan blue cultures were treated with potassium permanganate neither the green color of the bile masses nor the blue color of the stained cytoplasmic granules had shown any change three and a half hours later, when the observations were discontinued. Similar bright green granules and masses were found in the mesenchymal cells of a few cultures made from the gall-bladder of a nine-day embryo. The explants and the debris in these cultures were also bright green.

#### *Other cytoplasmic granules*

Other granules were found in both the endoplasm and ectoplasm and also in vacuoles when these were present. Many of them seemed to lie in the transition region between the ectoplasm and endoplasm where they moved about very actively. These bodies were of various shapes and easily distinguishable from the mitochondria by their different refractivity, by their rough irregular contour, and by their affinity for neutral red and trypan blue (figs. 9, 10, 11, 12, 13, 14, 17, 18). They varied in size from granules barely visible with the highest magnifications to masses as large as the nucleolus and in a few cases as large as the nucleus. Their form was very different from that of the corresponding granules found in the mesenchyme cells, being peculiarly angular and irregular. The smaller granules moved about very actively, often passing swiftly along the entire edge of the cells, or the full length of their enclosure. For this reason they were easily seen without staining. The larger bodies moved less rapidly and less extensively. These granules were observed in cultures from embryos of from five to twelve days' incubation. In a few cultures they were not present in the first cells which projected from the edge of the explant, but in most cases they were found in all stages of cultivation from the appearance of the first migrating cell to the death of the culture, in some instances as long as nine days after planting. In early stages of incubation the granules were few and small, increasing in both number and size as cultivation progressed until, in cultures four or more days old, they were often large and numerous (figs. 11, 17).

When neutral red was added to the culture medium these bodies took it up at once, becoming a bright yellow-red within a few seconds after the addition of the dye. If the solution of neutral red was very weak their progressive staining could be watched. Other granules not visible in the unstained cell also appeared at the same time as yellow-red bodies in the ectoplasm or in the endoplasm among the unstained mitochondria. Before the dye was added it was not possible to distinguish all the granules scattered among the mitochondria nor to see nearly so many granules in the ectoplasm or intermediate region. It is believed, however, that neutral red is only taken up by pre-existing granules and that, although some of them were not seen because of their unfavorable position, all of the granules were present before the neutral red was added. Those which appeared after the addition of the neutral red had the same characteristics of size, shape, and behavior as the granules which were visible before staining. Their staining reaction was identical and they had much the same distribution. Moreover, they increased in size and number with the aging of the culture, as was the case with the unstained granules.

When neutral red was added to the culture medium at the time the culture was made, the first cells to project from the explant did not contain any unstained granules but they almost always contained a few small yellow-red granules. These showed the same characteristics of shape, size, distribution, and behavior as the unstained granules in ordinary cultures and the granules in the cells stained supra-vitally with neutral red. As the neutral red cultures aged the granules increased in both size and number until in some cultures several days old they nearly filled the cell and completely hid the mitochondria, giving the same picture as the older cultures stained supra-vitally with neutral red. This correlation between the age of culture and the size and number of granules could be best worked out in those cultures grown in media with the dye, as the same culture could be noted at different stages, whereas in supra-vital staining comparisons are dependent upon different cultures at different ages, individual differences affecting the results to some extent.

When trypan blue was added to the medium at the time of planting, deep blue granules were seen in all the cells from the time of their first appearance. As trypan blue enters the cells very slowly, it cannot be successfully used supra-vitally and staining of the granules by it cannot be watched as in the case of neutral red. It is therefore difficult, perhaps impossible, to be sure always that the trypan blue granules are pre-existing granules to which the dye has become attached. We believe, however, that the trypan blue, like the neutral red, is deposited only in pre-existing granules. The granules stained by trypan blue resembled the neutral red granules in every way. When explants from the same embryo were cultivated in the same medium, except that one lot contained trypan blue and the other lot neutral red, the blue and the red granules were alike in shape, distribution and behavior. In size and number there was often some difference; the trypan blue cultures were usually 18 to 24 hours ahead of unstained or neutral red cultures in rate of growth, and showed more and larger granules than the neutral red cultures of the same age.

When explants were cultivated in media containing both neutral red and trypan blue, there appeared in the cells granules varying in color from a rose-red to a purple, depending on the relative amounts of the two dyes present. These granules had the same characteristics of shape, size, distribution and behavior as the yellow-red granules of the neutral-red cultures and the blue granules of the trypan-blue cultures. Three sets of cultures from the same embryo were made at the same time, one with neutral red, one with trypan blue, and one with trypan blue and neutral red in the media. The red, the blue and the lavender granules of the three sets of cultures at a given age were alike in every way except in number and size; the trypan-blue granules usually exceeded the others in these respects; the doubly-stained ones were second, and the neutral red the smallest and fewest. When a very dilute solution of neutral red was added to a trypan-blue culture no yellow-red granules appeared, but the blue granules could be seen to change to purple, then to lavender, and, if enough neutral red were present or if more were added, to a



rose-red, very different from the yellow-red color they exhibited when stained with neutral red alone. These color changes occurred first in the cells at the very edge of the growth and progressed inward toward the explant, which finally became tinged with the neutral red, usually, however, not going beyond the purple stage. The granules in all the cells of the culture showed these color changes. The granules in the endothelial cells showed the same degrees of staining with the two dyes. The wandering cells usually took up more of the neutral red than the other cells, their granules being rose-red when the granules of the other cells were lavender, and lavender when the others were purple.

When the cells of such doubly-stained cultures died, the neutral red color disappeared first, the trypan blue later, so that in a degenerating culture there might be at the same time cells showing lavender granules, others showing blue granules, and debris which was colorless. Much the same effect was obtained when the cultures were treated with potassium permanganate. Blebs often appeared very soon after the permanganate was added to the culture, and the red color was gradually lost, sometimes being entirely gone after two hours, at which time the blue staining had not usually been affected. Often it was not possible to recognize colorless granules after the neutral red or trypan blue had disappeared from the cell, probably owing either to their disintegration or to the generally contracted, degenerate condition of the cell.

The distribution of these cytoplasmic granules was not at all constant. Although most young cultures showed them, not all did; nor did all the cells of a culture behave similarly in this respect. Frequently one liver cell would show granules while a neighboring cell showed none at all (figs. 12a, 12b). When vacuoles were present in the cells some of them always contained one or more granules in active Brownian movement, while in some vacuoles no granules were visible (figs. 2, 9, 10, 11, 17a, 17b). In the marginal cells the greater number of the granules usually lay in the distal portion of the cell (fig. 9), but this was probably merely the result of the position of the nucleus which was usually

near the base of the cell, most of the cytoplasm being therefore in the distal part. A few granules often occurred in or near the base of the cell. Frequently there was an accumulation of the granules in some one part of the cell (figs. 11, 13); the position of this accumulation however, was not constant, and the granules were also often found scattered throughout the cytoplasm (figs. 9, 10, 14, 18). Fat globules and mitochondria were present in usual amounts. These granules seem to be similar in all respects to the granules described in the fibroblasts of cultures by Dr. Lewis<sup>3</sup> and called by him *degeneration granules*. As in the fibroblasts, they tend to accumulate in the liver cells in proportion to the age of the culture and are most numerous in the cells which are degenerating.

#### *Fat*

Free fat globules derived from the explants were present in the media of all the cultures and were distributed throughout the entire drop. They were especially abundant about the explant and diminished sharply in number as their distance from the explant increased (fig. 15). Considerable correlation was found between the ages of the embryos from which the explants were taken and the amount of free fat present in the medium. Increasing amounts of free fat globules accumulated in the media with the increasing age of the embryo from which the explants were taken. Explants from five day embryos gave up very little fat, while explants of sixteen day embryos gave up an enormous amount (figs. 3, 15). Occasionally the number of fat globules was so great as to make impossible the examination of the liver cells. After the first twenty-four hours of cultivation there was no perceptible increase in the amount of free fat in the medium.

A similar correlation was found between the age of the embryo and the number and size of the fat globules in the liver cells of the growth (figs. 2, 3, 7, 12, 14, 16, 17, 18). The cells of cultures of five day embryos had very few fat globules, some none at all. The cells of cultures of six to twelve day embryos showed in-

<sup>3</sup> Lewis, W. H. 1919 Degeneration granules and vacuoles in the fibroblasts of chick embryos cultivated in vitro. Johns Hopkins Hosp. Bull., vol. 30, p. 81.

creasingly more fat (figs. 2, 7, 12, 14, 17, 18), and the cells of sixteen day embryos usually contained numerous large globules (fig. 16). There was no correlation between the amount of fat and the age of the culture. The number or size of the fat globules in the cells did not change as the culture aged.

### *Degeneration*

In general, degeneration of the liver cells took place either by vacuolization or by bleb formation. These two processes never occurred together in the same culture: a culture which degenerated by bleb formation never showed any vacuolization of its cells (fig. 18); on the other hand, the cells of a culture in which vacuoles were found never showed any tendency to form blebs (figs. 2, 10, 11, 17, 22, 23, 25).

In two cultures vacuoles were observed on the first day after planting; in one case the first vacuoles appeared on the eighth day after planting; in most cases they appeared on the third day. The first vacuoles were single and small, approaching the nucleolus in size, and were present in only a few cells of the culture. Vacuolization in these cultures was progressive in extent as well as in degree, not all the cells becoming vacuolated at once.

Most of the vacuoles observed were smoothly spherical in form and did not change in shape or position. Some, however, were very irregular and constantly changing form, extending first in one place and then in another and consequently shifting about considerably in the cell. In some cases the vacuoles merely bulged out in various places (figs. 2, 11); in others definite processes were thrown out and withdrawn (fig. 9b). Sometimes these processes were very long and slender, forming vacuolar channels similar to those described by Lewis and Lewis<sup>4</sup> and W. H. Lewis.<sup>3</sup>

The vacuoles and their processes stained with neutral red, varying in color from a very light pink to a red, according to the concentration of the solution. No staining with any of the trypan-blue solutions used was observed.

<sup>4</sup> Lewis, M. R. and W. H. 1915. Mitochondria and other cytoplasmic structures in tissue cultures. *Am. Jour. Anat.*, vol. XVII.

Granules in active Brownian movement were observed in most of the vacuoles; in a few none could be made out. The first granules to appear in the vacuoles were small and single; as the culture aged the granules within the vacuoles increased in size or in number, or both. In some older cultures a large number of small granules were sometimes present in a vacuole; in most, however, there were two or three large irregular masses to a vacuole. All of these bodies showed active Brownian motion as well as more extensive moving about within their enclosure.

The early vacuoles appeared in any part of the cell midway between the nucleus and the cell edge, in contact with neither. As they increased in number they remained grouped about the nucleus so that in the marginal cells there was a clear edge of normal appearing cytoplasm with a proximal mass of vacuoles surrounding the nucleus (fig. 25). Their increase in size and number continued until finally the whole cell was changed into a foamlike mass surrounding a more or less distorted nucleus (fig. 23). At this stage the cell stained very deeply with neutral red. Eventually the whole growth fragmented and broke loose from the coverslip, what was left of the degenerated cells being represented by small, granular, irregular masses no longer showing any signs of vacuolization and floating free in the medium.

In a few untreated degenerating cultures, and in those treated with potassium permanganate or janus green, the fluid in the degenerating cell did not form vacuoles throughout the cell substance but collected along the periphery, forming clear blebs. In the untreated cultures this form of degeneration occurred a little later than the average time for vacuolization; bleb formation was not observed in any untreated cultures before the fourth day of cultivation and many cultures did not show it before the seventh day. In some cultures treated with potassium permanganate blebs formed within five minutes after treatment; in those treated with janus green, blebs appeared in a half to one and a half hours after the addition of the dye.

The first indication of this type of degeneration was the contraction of the cells. A few minutes later clear spaces appeared

between contiguous cells. Very soon clear, blister-like sacs or blebs began to protrude from the free cell edges and were alternately retracted and extended from various parts of the free edge of the cell. These blebs often contained one or more granules in active movement. Meanwhile the boundaries between contiguous cells disappeared and the granular contents of the cell became greatly crowded. Granules in cells at this stage could be stained with neutral red and if previously colored with either neutral red or trypan blue, or both, still retained their color. After a varying length of time (never more than three hours) the neutral red color faded, and if more was added to the culture it was not taken up (figs. 18a, 18e). Very soon after the neutral red faded the blebs disappeared and the cells were left as irregular masses of shadowy granules surrounding more or less misshapen and compressed nuclei. The trypan blue staining was retained for a much longer time than the neutral red.

#### WANDERING CELLS

The wandering cells found in these cultures were usually small, contracted, irregular cells with many clear, pointed processes. Their cytoplasm was packed with fat globules which generally hid all other details. Sometimes a few of these cells were flattened; in these the clear nucleus and many angular granules of all sizes could be made out. In the wandering cells of some of the cultures irregular green masses were also present; these were interpreted as bile granules which the cells had taken up from the degenerating liver cells of the explant. In one culture from an 11 day embryo every wandering cell found on the second day of cultivation contained one or more bright green bodies.

These macrophagic cells occurred in considerable abundance in the cultures, especially in those which showed little or no growth of liver cells or endothelial cells. They were scattered all through and over the explant and growing edge, and usually many were free in the culture, isolated from the other cells and from each other. They moved about actively, throwing out and retracting their processes.

The granules in these cells showed the same color reactions as those of the liver cells, except that the colorless ones took the stains much more deeply, making the wandering cells the most prominent features of the cultures. The granules stained an intense red with neutral red, a deep blue with trypan blue, and a good purple or lavender when both dyes were present in the culture medium. They took up neutral red more readily than trypan blue when both were present, so that when the other cells had lavender granules the wandering cells had rose-red granules, or lavender granules when the other cells had purple ones. They were the last cells to lose their staining when the culture degenerated.

The green bile masses in the cells stained slowly but intensely with the neutral red, but apparently they did not take up the trypan blue. In trypan blue cultures both blue and green granules were present in the same wandering cell. In neutral red cultures all the granules were stained red.

These cells are much more satisfactory for study when stained with trypan blue than when stained with neutral red, as the granules when stained with the latter dye mask the fat globules and the nucleus to a much greater extent than when stained with the blue.

#### DEBRIS

In many liver cultures a large amount of debris accumulated, sometimes entirely obscuring the details of the growth. This debris occurred either as groups of long anastomosing threads stretching out from the edges of the explant and finally overgrowing the whole field, or as a fine granular or flocculent precipitate deposited all over the field. The long, rough, interlaced threads often had the granular debris caught in among them; sometimes long, single, isolated threads stretched out from the explant to a considerable distance. The two types stained alike, taking hemateine, hematoxylin and Gram's stain very deeply. In no other kind of cultures made in this laboratory has debris of this sort occurred so frequently or in such large amounts as in these liver cultures.

In twenty-eight cultures made from embryos varying from six to twelve days' incubation an unusually large amount of debris was present. These cultures included ten planted in media without any stain, nine planted in trypan blue media, eight planted in media containing both trypan blue and neutral red, and one in a neutral red medium. The cultures showing debris were usually discarded on the third day after planting as worthless for liver cell growth. They seldom showed growth of any kind, still more rarely any growth of liver cells. One culture showed a good growth of liver cells which was finally entirely hidden by debris.

In eight sets plants were made of the subcutaneous tissue of the same embryo from which the liver tissue was taken and planted at the same time in the same media, for comparison with the liver in regard to the occurrence of debris. These sets were made from embryos of seven to twelve days' incubation and the tissues were washed in three dishes of media before planting in order to remove all free blood. Sixty-three per cent of the liver cultures and ten per cent of the connective tissue cultures showed debris. In the liver cultures the debris, which was often present on the first day after planting, increased with the age of the culture. In the five connective tissue cultures in which it was present on the first day after planting, no increase in amount occurred as the culture aged; on the contrary, no debris was to be found on the second day after planting in any of these five cultures.

This debris behaves in many respects like fibrin but we are still uncertain as to its exact nature.

#### SUMMARY

Cultures were made from the liver of chick embryos from 5 to 18 days' incubation. The best growths were obtained from embryos of 5 to 12 days. No liver cells proper were obtained from embryos older than 16 days.

Four types of cells (liver cells, endothelial cells, mesothelial cells and wandering cells) migrated out from the explants in varying numbers; some cultures contained only one, or two, or three types.

The liver cells migrated out in the form of a membrane or sheet. No cell division was observed in them.

The liver cells showed ectoplasm and endoplasm, and in the latter were seen mitochondria, bile granules, neutral red granules and fat globules. The ectoplasm sometimes contained a few neutral red granules.

The mitochondria were very numerous and in the form of more or less irregular granules, varying in size even in the same cell.

Bile granules (bright green masses) were only occasionally seen in the liver cells. These showed a great affinity for neutral red and changed to orange, brown, reddish-brown and finally to a very dark red color when this dye was added to the culture medium.

The cytoplasmic or 'degeneration' granules varied in number; only a few were seen in the cells of young cultures. They gradually increased in number and size in the older cultures. A few were seen in the ectoplasm where their movements were more marked. They had a great affinity for neutral red and trypan blue, taking up the former rapidly, the latter slowly, and losing the neutral red rapidly and trypan blue slowly as the cells died. The granules took up the two dyes at the same time, the color varying between red and blue according to the relative strength of the dyes.

Neutral red and trypan blue seem to be taken up only by pre-existing granules and by the same granules.

Fat globules increased in number with age of the chick, but not with the age of the culture. They were seen in the liver cells and also free in the media.

Degeneration occurred either by vacuolization or by bleb formation.

The endothelial cells formed the usual loose, reticular-like outgrowths. Since the embryonic liver contains little or no mesenchyme, it is very probable that many of the so-called mesenchyme cells were from the endothelium of the sinusoids.

Wandering cells occurred frequently and often contained bile granules.

Debris (or fibrin?) from the explant often occurred in large amounts, at times obscuring the outgrowths.



## PLATE 1

### EXPLANATION OF FIGURES

1a-1f Six views of a culture, explant from a 16 day embryo. No growth on the third day.  $\times 37$ .

1a Fourth day of cultivation; 12.00 Noon. Explant and four small groups of migrated liver cells.

1b Fifth day of cultivation; 10.30 A.M. Growth membrane of considerable size around almost the entire edge of the explant. Fifteen wandering cells have migrated out from the explant.

1c Fifth day of cultivation; 3.00 P.M. The growth membrane has changed in shape but has not appreciably increased in size. Outlines of a few cells and their nuclei are shown; fourteen wandering cells present.

1d Sixth day of cultivation; 11 A.M. The growth membrane had become much more ragged and fenestrated.

1e Sixth day of cultivation; 4 P.M. One isolated group of liver cells. Fourteen wandering cells.

1f Eighth day of cultivation. The growth membrane is very much broken up; many isolated cells and many dead cells.

2a-2h Eight views of a cell on the fourth day of cultivation, explant from a nine day embryo, showing the rapid and extensive changes in form of a large vacuole containing granules. The small spherical vacuoles did not change shape. Views 2a-2e are of the unstained cell; 2f, of the cell stained with janus green; 2h, of the cell after neutral red staining.  $\times 750$ .

2a 3.34 P.M. Showing cell outlines, nucleus, nucleolus, the fat globule, a few of the mitochondria, and the vacuoles, some of them containing granules.  $\times 750$ .

2b 3.40 P.M. All of the mitochondria are shown here.

2c 3.47 P.M.

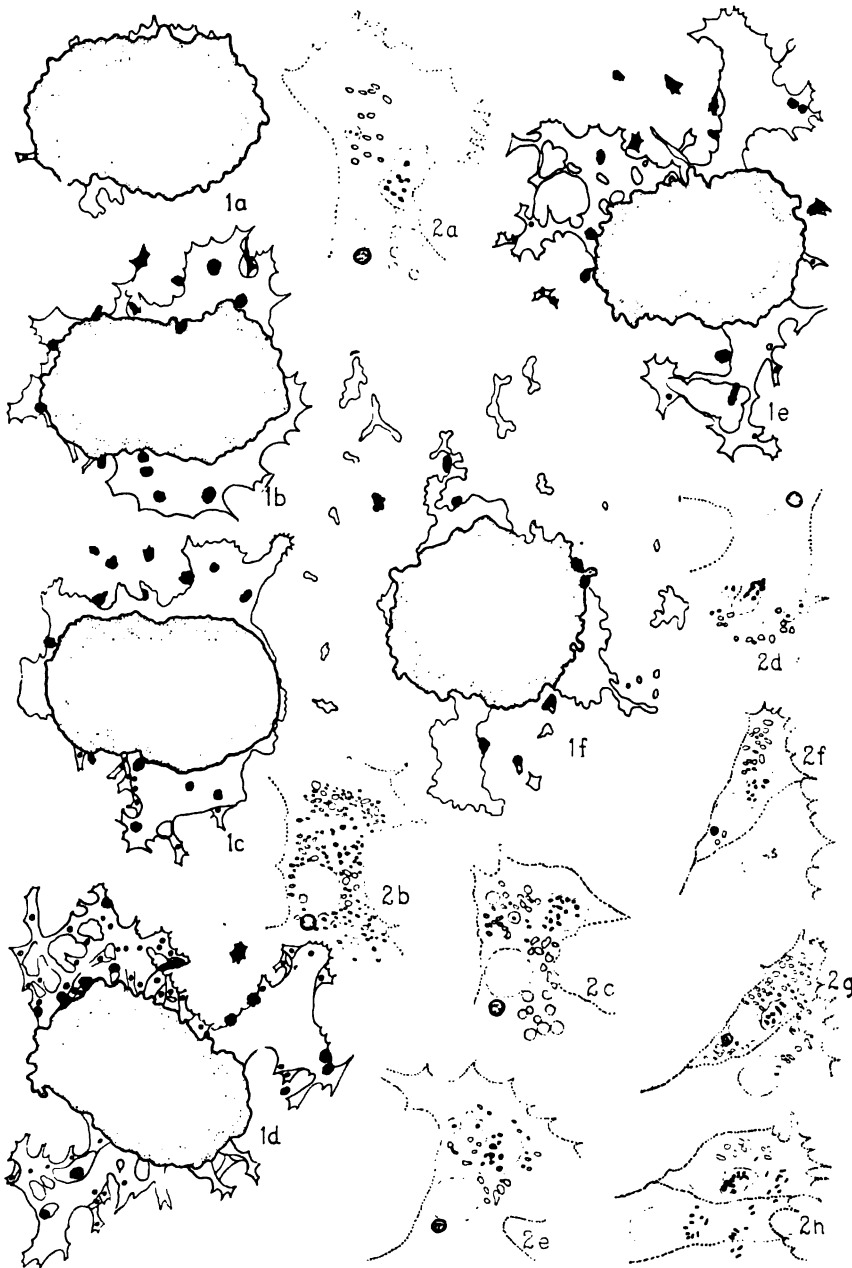
2d 3.58 P.M. The vacuole has broken up into two smaller vacuoles, each containing granules.

2e 4.05 P.M. The two vacuoles have united.

2f 4.20 P.M. Janus green added. The culture contracted with a consequent decrease in extent of area of cell, nucleus and vacuole. None of the smaller vacuoles are to be seen. Another cell has grown out behind the original cell.

2g 4.21 P.M.

2h 4.29 P.M. After neutral red has been added to the culture. A third cell has begun to grow out and in the three cells numerous granules free in the cytoplasm, which were not before observed, are now visible.



## PLATE 2

### EXPLANATION OF FIGURES

3a-3c Three views of the upper edge of the explant shown in figure 1a, 3a the edge at 12.00 noon; 3b at 2.25 P.M.; and 3c at 2.30 P.M. The outlines of the individual liver cells, the nuclei, the fat globules, many free in medium, and one clasmatocyte are shown.  $\times 312$ .

4a-4q Seventeen views of a group of liver cells along the edge of an explant from an eight day embryo on the second day of cultivation. On the fourth day of cultivation this plant had given rise to an excellent liver cell growth.  $\times 312$ .

4a 2.00 P.M. Showing the two cells (numbered 4 and 5) which had migrated out from the explant at this point.

4b 2.30 P.M. Showing the four additional cells which had grown out in the intervening half hour. (Numbers 1, 2, 3, and 6.)

4c-4j. 2.40-3.55 P.M. Eight views drawn at ten minute intervals showing the movements of these six cells.

4k 4.00 P.M. A seventh cell has grown out.

4l-4q 4.15-4.55 P.M. Six views of the seven cells at the following intervals: 15 min., 10 min., 10 min., 2 min., 3 min.

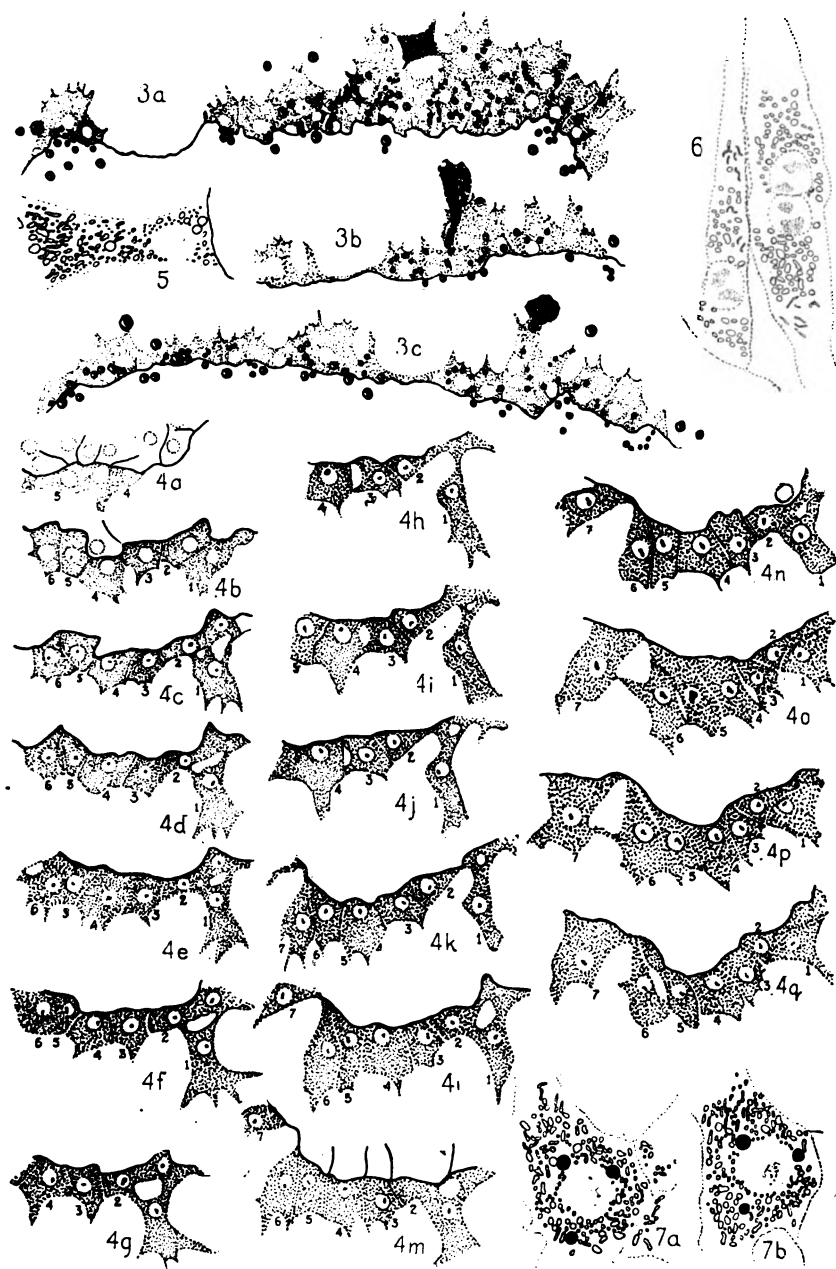
5 A single migrating liver cell with its nucleus and mitochondrial granules on the second day of cultivation; explant from a ten day embryo. The limits of the outer edge of the cell were not visible.  $\times 750$ .

6 Two migrating liver cells, explant from an eight day embryo; 3 day cul. showing nuclei each with two nucleoli and mitochondria. One cell is binucleate and has many more mitochondria than the other.  $\times 750$ .

7 Two views of a liver cell, explant from a 10 day embryo, five day culture.  $\times 750$ .

7a 11.26 A.M. Showing nucleus, nucleoli, mitochondria and three fat globules.

7b 11.34 A.M. The same, eight minutes later.



### PLATE 3

#### EXPLANATION OF FIGURES

8 Two liver cells on the third day of cultivation, explant from a nine day embryo. Showing differences in mitochondrial content in cells side by side.  $\times 750$ .

9a-9b Two views of a liver cell on the third day of cultivation, explant from a seven day embryo.  $\times 750$ .

9a Showing cell outlines, nucleus, nucleolus, mitochondria and ten colorless cytoplasmic granules (black in figure).

9b The same cell after the addition of neutral red. A few more granules were visible stained a deep yellow-red. At X an irregular vacuole also stained, from which stretch out neutral red canals; these were continually retracted and extended, the two pointing toward the base of the cell being particularly active.

10 A liver cell on the fifth day of cultivation, explant from a nine day embryo, stained with neutral red. Showing cell outlines, position of nucleus, mitochondria, granules (black in figure), and vacuoles (dotted circles), some of the latter containing granules.  $\times 750$ .

11 A liver cell with neutral red added to the medium at the time of observation; five day culture, explant from a seven day embryo; showing nucleus, nucleolus, granules and vacuoles. Two of the granules are large, the others small; a few lie within one of the vacuoles. The two large vacuoles in this cell changed form continually. The mitochondria have not been drawn.  $\times 750$ .

12a-12b Two liver cells or cell-groups on the fifth day of cultivation, explant from a nine day embryo, stained with janus green and neutral red, and showing nuclei, nucleoli, mitochondria, neutral red granules and fat. In each group there are three nuclei, no cell boundaries visible between them. In cell a no neutral red granules were visible; in cell b there were 4 granules in one part of the cell. There are three nucleoli in one nucleus of cell b.  $\times 750$ .

13 A liver cell on the fifth day of cultivation, explant from a six day embryo; neutral red added to medium at time of planting. Showing nucleus with three nucleoli, two fat globules, mitochondria, four scattered neutral red granules and two groups of granules, one small and one large.  $\times 750$ .

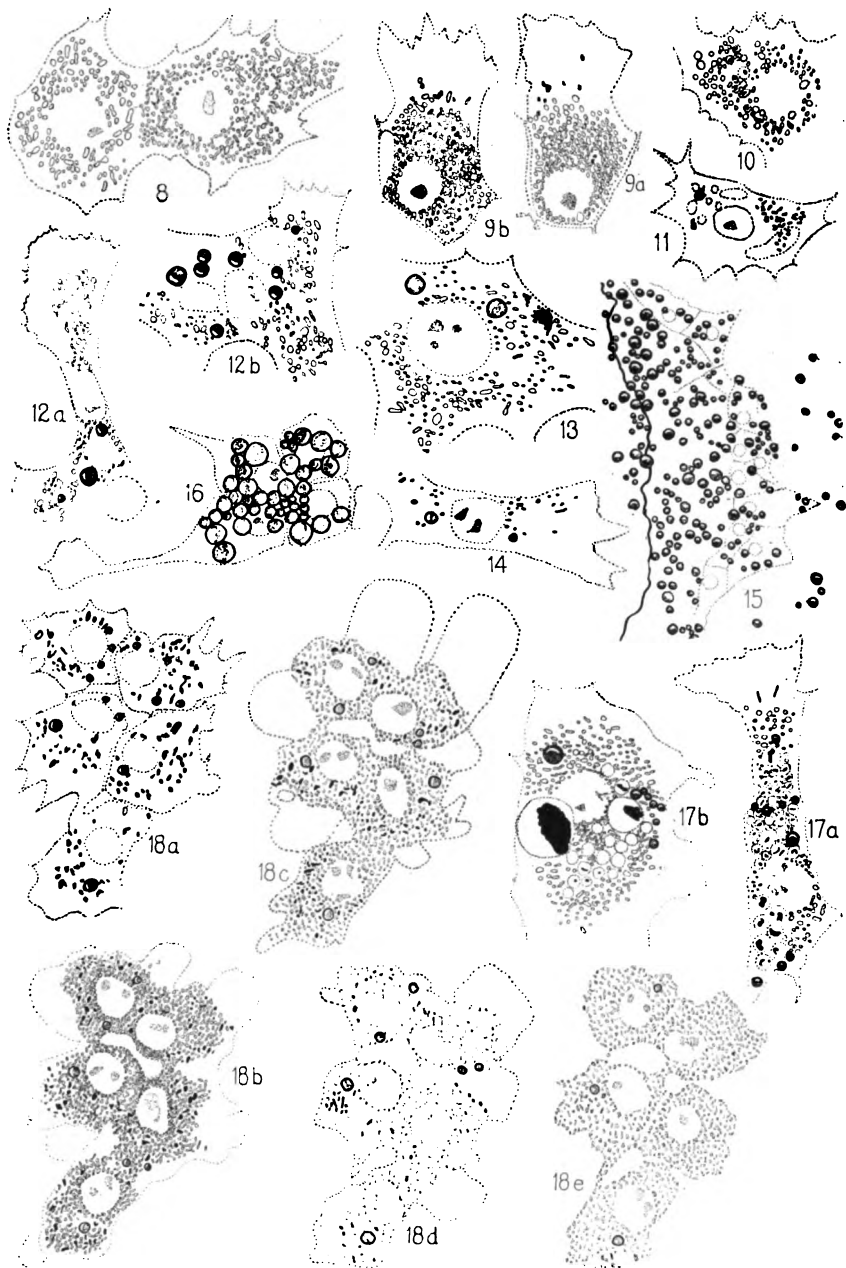
14 A liver cell on the second day of cultivation, explant from an eight day embryo; trypan blue added to medium at the time of planting; showing nucleus with two nucleoli, two fat globules and many trypan blue granules.  $\times 750$ .

15 Portion of a liver cell membrane on the third day of cultivation, explant from a 16 day embryo; edge of explant indicated by heavy line; cells at edge of explant in dotted lines. Many fat globules, most of them free in the culture medium derived from the explant.  $\times 312$ .

16 A group of four liver cells on the seventh day of cultivation, explant from a sixteen day embryo, showing cell outlines, nuclei, two nucleoli, and intracellular fat globules. The fat globules are numerous and large, approaching the nuclei in size.  $\times 750$ .

17a-17b Two liver cells on the ninth day of cultivation, explant from an 8 day embryo; trypan blue in the medium.  $\times 750$ .

(Continued on page 306)



(Continued from page 304)

17a Nucleus with 4 nucleoli, nine fat globules, mitochondria, vacuoles with and without blue granules (black in figure).

17b Contains two very large vacuoles with very large blue granules.

18a-18e Five views of a group of five cells on the seventh day of cultivation, explant from a ten day embryo; trypan blue in medium, supra-vitally stained in neutral red. Showing degeneration by bleb formation.  $\times 750$ .

18a 12.45 P.M. Showing cell outlines, nuclei, fat globules, and angular rose-red stained granules (black in figure). Spaces have developed between contiguous cells, mitochondria not shown.

18b 1.25 P.M. The mitochondria and nucleoli are shown in this view in addition to the granules. Cell boundaries have disappeared, and bleb formation has begun.

18c 1.45 P.M. The blebs are much larger, and the mitochondria have lost their clear cut outlines and have become indistinct granular bodies. The rose-granules are still well stained.

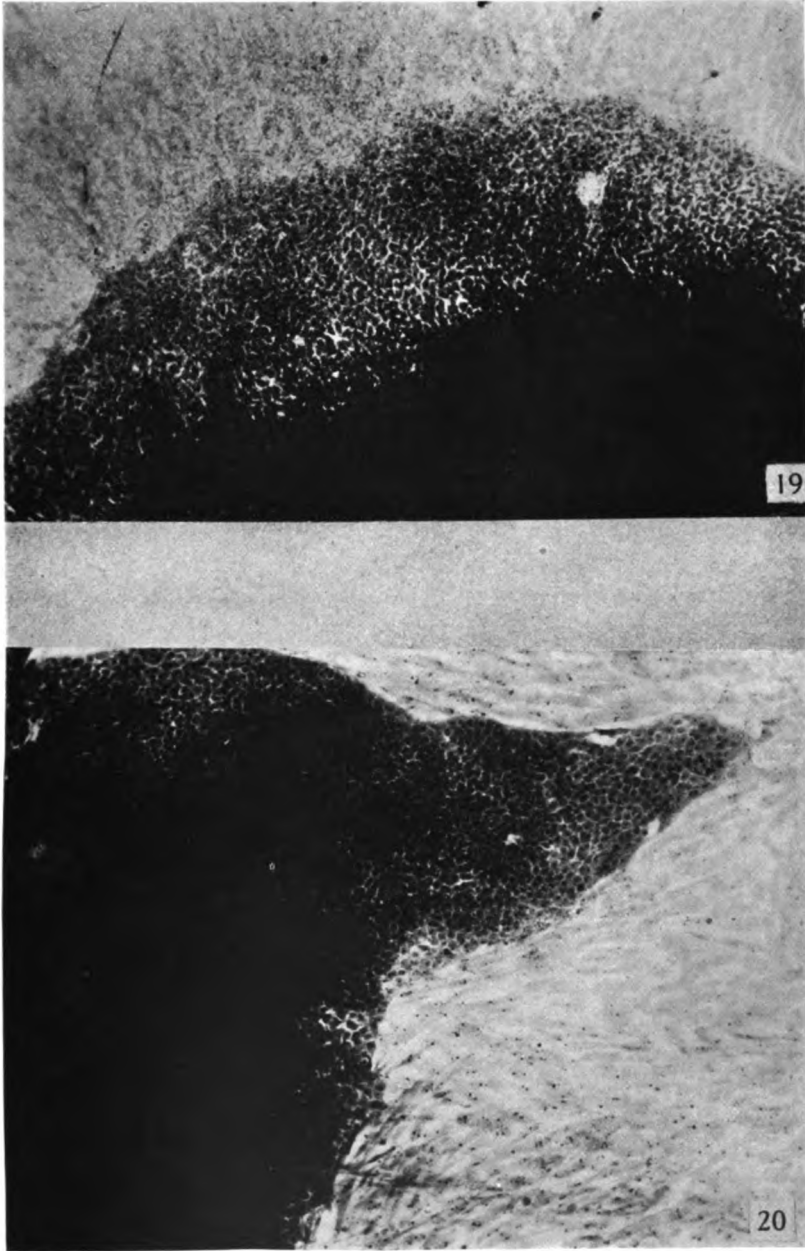
18d 2.45 P.M. The rose color has disappeared from the granules leaving them a bluish-gray color. Neutral red has been again added, but the granules have not taken it up, retaining the bluish-gray color. The mitochondria have become still more indistinct and the blebs have decreased in size and number.

18e 3.45 P.M. Bleb formation has entirely disappeared and the cells are merely accumulations of granular masses representing the mitochondria, with a few darker bodies among them, the granules which still retain some of their trypan-blue staining.

## PLATE 4

### EXPLANATION OF FIGURES

19 and 20 From 6 day culture, explants from 8 day embryo. Liver cell membrane and endothelial reticulum. Fixed in Schaudinn's fluid, iron haematoxylin.  $\times 125$ .



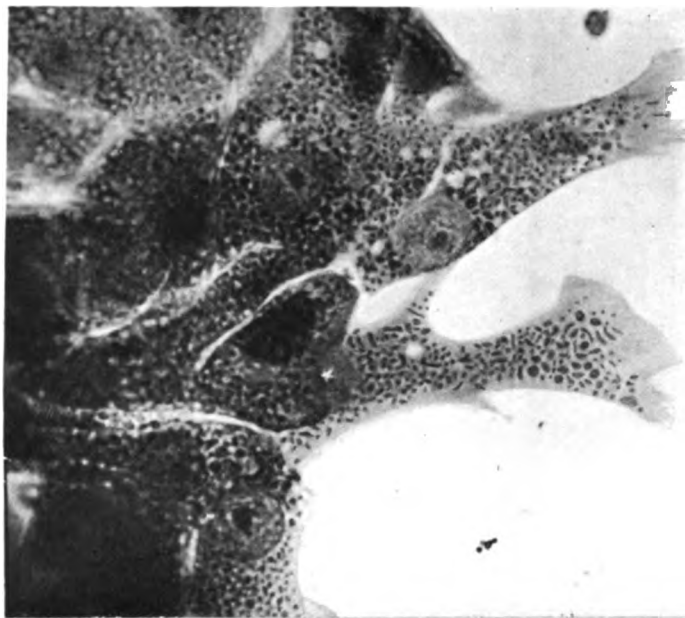


## PLATE 5

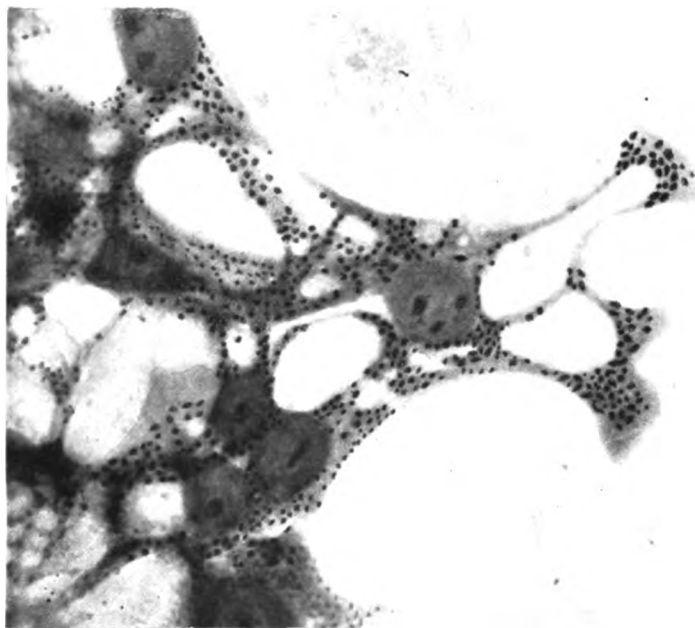
### EXPLANATION OF FIGURES

21 A few of the liver cells from a five day culture, explant from a ten day embryo, fixed in Zenker's, Bensley stain. Cell outlines, nuclei, nucleoli and mitochondria are shown.  $\times 1320$ .

22 Highly vacuolated liver cells, 4 day culture, explant from an 8 day embryo, fixed in Zenker's, Bensley's stain. The nuclei and the nucleoli are shown, the large and small vacuoles, the deeply stained mitochondria lying in the cell edges and the cytoplasmic strands between the vacuoles.  $\times 1320$ .



21



22

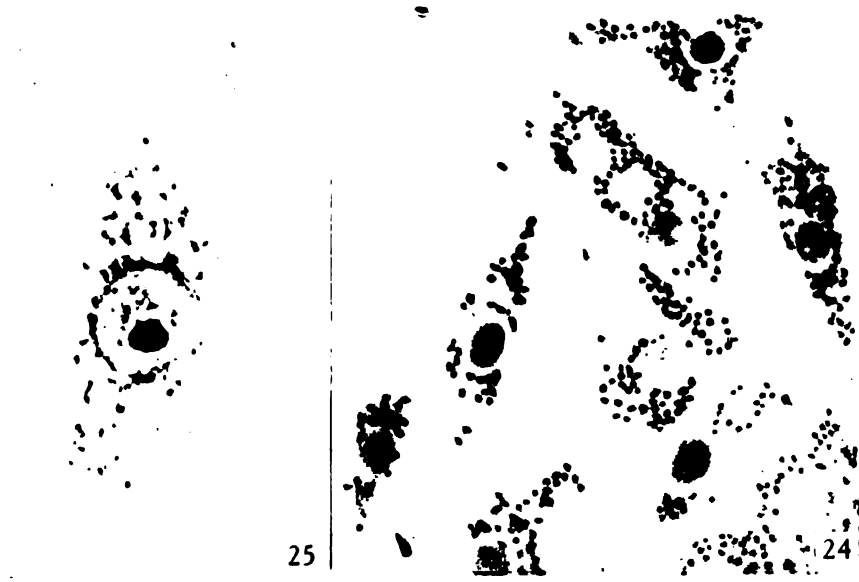
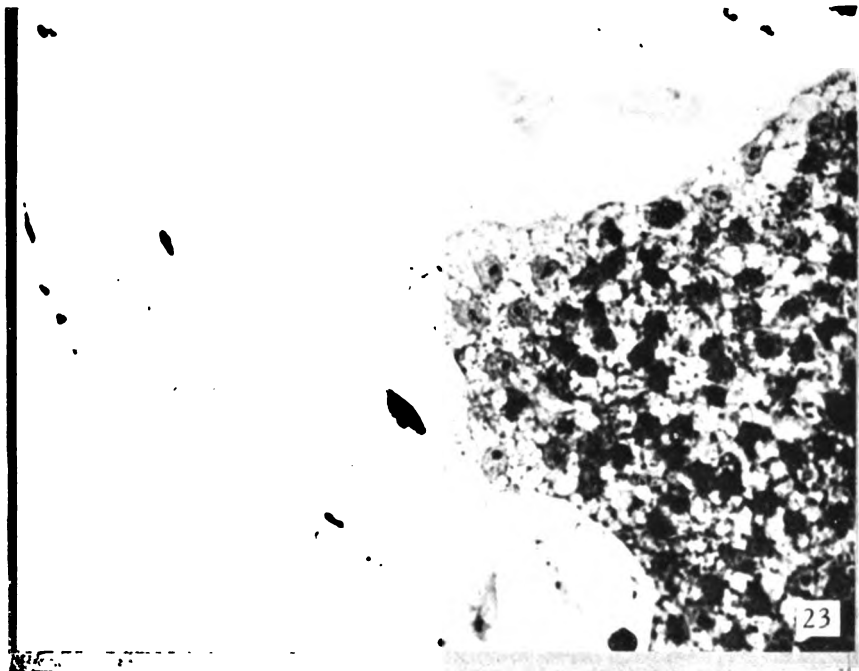
## PLATE 6

### EXPLANATION OF FIGURES

23 Vacuolated liver cell membrane, a few endothelial cells; 6 day culture, explant from 8 day embryo; Zenker, iron haematoxylin.  $\times 660$ .

24 Isolated liver cells. 4 day culture, explant from 8 day embryo; osmic acid vapor, hemateine.  $\times 1320$ .

25 Isolated liver cell highly vacuolated, few mitochondria in the meshes between vacuoles. 6 day culture, explant from 9 day embryo.  $\times 1320$ .





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Resumen por los autores, G. B. Wislocki y T. J. Putnam.

La absorción en los ventrículos de hidrocéfalos internos producidos experimentalmente.

Los autores han producido hidrocéfalos en algunos gatos y conejos jóvenes mediante inyección de una suspensión de negro de humo en las cisterna cerebello-medular. Más tarde inyectaron en los ventrículos dilatados una solución muy difusible o un colorante coloidal. Los experimentos demuestran que la absorción se lleva a cabo con cierta intensidad en los animales hidrocéfalos desde los ventrículos, y que la ruta seguida es a través del epéndimo, penetrando en los espacios intercelulares y finalmente en los espacios perivascuales. La rapidez de difusión de una verdadera solución (ferrocianuro potásico y citrato ferro-amónico) desde las cámaras cerebrales hasta la sustancia cerebral es bastante rápida, pero la de una solución coloidal (azul tripan) es algo más lenta. Los autores no han llevado a cabo observaciones sobre la rapidez de la absorción de sustancias extrañas por los capilares. Tampoco han encontrado prueba alguna sobre la absorción por los plexos coroides.

Translation by José F. Nonides  
Cornell Medical College, New York

## ABSORPTION FROM THE VENTRICLES IN EXPERI- MENTALLY PRODUCED INTERNAL HYDROCEPHALUS

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### FOUR FIGURES

Internal hydrocephalus has been produced experimentally in several ways. Dandy and Blackfan ('13, '14) reported the successful production of an obstructive hydrocephalus in dogs following the introduction of pledgets of cotton into the aqueduct of Sylvius. Thomas ('14) described an internal hydrocephalus produced in dogs by the intraventricular injection of aleuronate. The aleuronate caused a sterile inflammatory reaction which occluded the ventricular cavities at one or more points and prevented the escape of the cerebrospinal fluid into the subarachnoid space. Subsequently, Dandy ('19) produced a communicating type of hydrocephalus by placing strips of gauze, saturated with iodine, about the mesencephalon.

Recently, Weed ('20) described the results of his experiments on a series of young and adult cats. He was able to produce an internal hydrocephalus with great facility by injecting a suspension of lampblack into the cisterna cerebellomedullaris through a puncture in the occipito-atlantoid ligament. It was found that the lampblack caused a sterile meningitis about the rhombencephalon, thereby gradually occluding the foramina in the roof of the fourth ventricle and preventing the escape of cerebrospinal fluid into the subarachnoid space. Hydrocephalus was also produced by intraventricular injections of the lamp black.

Weed's experiments on kittens in which the fontanelles had not united are mainly of interest. In these animals hydrocephalus developed extremely rapidly and the dilatation of the ventricles



and enlargement of the head were quite remarkable. An extreme dilatation of the ventricles and thinning of the cortex were frequently observed within ten days.

The present study was undertaken to determine whether substances injected into the dilated and occluded ventricles undergo absorption and, if they do, how they escape from the ventricles into the tissues of the brain. This question has to some extent been considered by several of the previous investigators. Dandy and Blackfan ('13, '17) described the results of injecting phenol-sulphonaphthalein into the ventricles of fifteen human beings suffering from internal hydrocephalus of the obstructive type. After injection the time of appearance and the amount of the drug in the urine were noted. They found that after injection of phenol-sulphonaphthalein into the dilated ventricles from 0.5 to 2 per cent of the drug was excreted in the urine within the first two hours. Excretion of the dye commenced from 20 to 45 minutes after injection and continued for many days. Their conclusion is that in obstructive hydrocephalus practically no absorption takes place from the ventricles.

In the present study hydrocephalus was established experimentally in animals, and several substances, the absorption of which could be studied histologically, were subsequently injected into the dilated ventricles. A solution of potassium ferrocyanide and iron ammonium citrate, which can be readily precipitated as Prussian blue in the tissues, and trypan blue, were selected for injection into the dilated ventricles. The method chosen for producing the hydrocephalus was the one described by Weed because the technique is relatively simple and hydrocephalus develops promptly.

The experiments were performed upon kittens and young rabbits. A total of twenty-five animals was used, consisting of fifteen kittens and ten rabbits. The kittens ranged in age from two to six weeks, the rabbits from one to three weeks. Weed's technique was closely adhered to. A 5 to 10 per cent suspension of lampblack in physiological salt solution was prepared. The animals were anesthetized, the skin shaved, and a needle inserted through the occipito-atlantoid ligament into the cisterna cere-

bellomedullaris. The kittens received  $1\frac{1}{2}$  cc., the rabbits  $\frac{1}{2}$  cc. of lampblack suspension. The immediate mortality from this procedure was rather high and due, as far as could be ascertained, either to injury to the rhombencephalon or to respiratory failure from the sudden increase in pressure on the medulla.

The animals surviving the injection recovered from either and appeared normal in all respects. The day following the injection nothing unusual could be observed. The first signs of a developing hydrocephalus could be made out on the second, more often on the third or fourth day. Gradually the enlargement of the cranial vault became noticeable and a diastasis of the cranial sutures could be made out. The animals' behavior changed somewhat. Slight ataxia was nearly always noted and tremors were observed in several instances. The injected animals appeared to be less active than the normal controls.

Several deaths occurred during the first ten days, some from intercurrent pneumonias, others from undetermined causes. On the tenth day eight kittens and four rabbits with well-developed hydrocephalus were alive and in good condition. These were used for the injection of the ventricles.

The animals were divided into two groups, one group receiving a 2 per cent solution of equal parts of potassium ferrocyanide and iron ammonium citrate, the other group a  $\frac{1}{10}$  per cent solution of trypan blue. The experimental procedure was as follows: The skin of the head was shaved and cleansed, a sharp, 26-gauge needle was inserted through the skin and bone into one of the lateral ventricles, and two drops of fluid were permitted to escape, whereupon an antitoxin syringe was connected with the needle and an equal quantity of potassium ferrocyanide and iron ammonium citrate or trypan blue was injected. The needle was removed and the animal released. The animals receiving potassium ferrocyanide and iron ammonium citrate were killed at intervals varying from thirty minutes to two hours, those receiving trypan blue at intervals from eight to twenty-four hours. The brains injected with potassium ferrocyanide and iron ammonium citrate were fixed in 10 per cent formalin plus 1 per cent hydrochloric acid. The acid precipitated the ferro-

cyanide and citrate solutions as Prussian blue. The brains which had been injected with trypan blue were fixed in 10 per cent formalin without the addition of acid. The fixation was accomplished by immersion, in order to avoid the disturbing factors of intra-arterial injection.

On gross inspection, the surfaces of the brains were found either unstained or very faintly colored. On cutting thin coronal

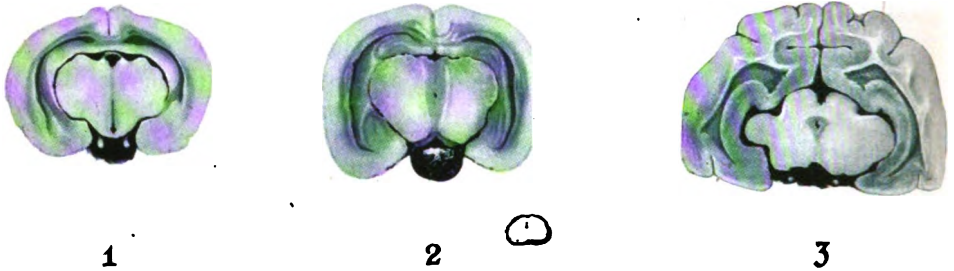


Fig. 1 Cross-section of the brain of a young rabbit in which dilatation of the ventricles had resulted from the injection of lampblack into the cisterna cerebello-medullaris. Subsequently a solution of potassium ferrocyanide and iron ammonium citrate was injected by replacement into a lateral ventricle. The animal was killed two hours later and the brain fixed. A zone of Prussian-blue-stained brain tissue can be seen surrounding the ventricles.

Fig. 2 Cross-section of the brain of a young rabbit in which dilatation of the ventricles has been similarly brought about. Trypan blue was injected into the ventricles of this animal. A zone of stained tissue can be seen surrounding the ventricles. The insert shows a section of the lumbar cord from the same animal. The wall of the central canal and a zone of tissue surrounding it are faintly stained.

Fig. 3 Cross-section of the brain of a young cat, showing the dilated ventricles into which a solution of potassium ferrocyanide and iron ammonium citrate was injected. A zone of Prussian-blue-stained tissue is seen surrounding the ventricles.

sections of the brains and cords a striking appearance was disclosed. The ventricles, as was expected, showed varying degrees of distention. The walls of the ventricles and a zone of surrounding brain tissue to a depth of several millimeters were deeply stained (figs. 1, 2, and 3). This zone of staining enclosed not only the lateral and third ventricles, but the aqueduct of Sylvius, the fourth ventricle and central canal of the cord, demonstrating a free communication between all of the cerebral ventricles and

the central canal. The inner portion of the zone of staining adjacent to the ventricles was most deeply colored, while towards the surface of the brain the stain became gradually paler. It is noteworthy that the stains had penetrated even into the most caudal recess of the central canal of the cord (fig. 2).

Microscopically, the dyes were readily visible in the tissues of the brain. Precipitated Prussian-blue granules were found in the tissues surrounding the ventricles. The precipitate occurred in the intercellular spaces, in the perineuronal spaces and to a large extent in the perivascular sheaths. Prussian blue was precipitated in minute granules in the cytoplasm of the ependymal cells. None of the precipitate was found to be intracellular, either in glial or nerve cells. The choroid plexuses did not appear to have been penetrated by the coloring matter.

The distribution of trypan blue differed microscopically somewhat from that of the Prussian blue. The choroid plexuses appeared entirely unstained. Precipitated trypan blue was visible on the surface of the ependymal cells in some areas, but none was discernible in the cytoplasm of the cells. Trypan blue could not be seen in the intercellular spaces or in the perineuronal spaces. Many of the glial cells contained dust-like particles of dye within their cytoplasm. Trypan blue was not visible in the cytoplasm of any of the nerve cells. The small vessels and capillaries in the zone of staining were noteworthy in the trypan-blue animals. Brilliantly stained cells containing numerous granules of the dye in their cytoplasm everywhere surrounded the blood-vessels (fig. 4). These cells which closely invest the blood-vessels belong to the category of adventitial cells or macrophages. They are extremely phagocytic and are the scavenger cells of the brain tissue, which have been repeatedly shown to play a prominent rôle in brain injury and repair.

The microscopic distribution of these substances indicates that they have followed definite pathways in their escape from the dilated ventricles. The solutions are observed in their escape through the ependyma into the intercellular spaces and thence into the perivascular sheaths. These observations lead to the conclusion that fluid currents exist between the dilated ventricles

and the neighboring vessels, under the abnormal conditions of a heightened intraventricular pressure.

It remains to be pointed out that there is no evidence microscopically that the substances behave as a fixative would if similarly injected. Neither of the reagents employed in these experiments appears to exert any deleterious influence on the cells which it encounters during its passage into the brain tissues. Nuclear staining or diffuse discoloration of the brain tissue, which would be indicative of cell injury and death, are not



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Fig. 4 Microscopic section of the cortex of the left hemisphere of a rabbit in which trypan blue had been injected into the dilated ventricles. A portion of a small cerebral vessel is shown, surrounding which are several phagocytic cells containing granules of trypan blue.

observed. The phenomena of distribution of the dye granules must be looked upon not as the result of diffusion of a foreign chemical through dead tissue, but as a true passage of the dyes through the brain tissue during life. The explanation for the different findings with the ferrocyanide-citrate solution and with the trypan blue lies in the fact that microscopic identification of the former requires only the action of the mineral acid, while vital activity on the part of a certain class of phagocytic cells is necessary for the intracellular aggregation of the vital-dye particles before microscopic identification is possible. As illustrating microscopically the pathway of absorption, the

ferrocyanide-citrate method possesses the advantage of freedom from the modifying influences of cell activity; the findings with trypan blue, however, in these experiments indicate the ultimate, if not the total, pathway of absorption of a colloidal suspension from the dilated cerebral ventricle.

#### SUMMARY

Hydrocephalus was produced in a number of kittens and young rabbits by injecting a suspension of lampblack into the cisterna cerebellomedullaris. Subsequently a readily diffusible solution or a colloidal dye was injected into the dilated ventricles. The experiments show that absorption occurs to some extent from the ventricles in hydrocephalic animals and that the pathway of escape is through the ependyma into the intercellular spaces and finally into the perivascular spaces. The rate of diffusion of a true solution (potassium ferrocyanide and iron ammonium citrate) from the cerebral chambers into the brain substance is fairly rapid, but that of a colloidal suspension (trypan blue) is somewhat slower. No observations on the rapidity of absorption of the foreign substances into the cerebral capillaries were made. No evidence of absorption by the choroid plexuses was obtained.

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Resumen por el autor, T. H. Bast

Varios tipos de amitosis en las células óseas.

El estudio de las células óseas ha demostrado que la amitosis es el método normal y exclusivo de multiplicación de dichos elementos. La amitosis en el hueso constituye dos tipos, determinados por la forma del núcleo en vías de división y por la función y posición de los centrosomas. En el primer tipo el núcleo presenta forma de bizcocho y los centrosomas están situados en cualquiera de sus polos. En el segundo tipo el núcleo presenta forma de herradura y los centrosomas, que no se han separado, yacen en una escotadura de dicho órgano celular. La existencia de masas de citoplasma casi separadas de las células principales indica que también tiene lugar la fragmentación de la célula. Una revisión de la literatura sobre la amitosis, junto con observaciones en otros tejidos, demuestra que existe otro tipo de amitosis en el cual una estructura en forma de placa indica el plano de la división nuclear. Los centrosomas de este tipo parecen carecer de función o pueden faltar por completo. La literatura sobre esta cuestión puede clasificarse según las diversas teorías de la amitosis que han sido propuestas hasta la fecha.

Translation by José F. Nonidez  
Cornell Medical College, New York

## VARIOUS TYPES OF AMITOSIS IN BONE CELLS

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TWO TEXT FIGURES AND ONE PLATE (TWELVE FIGURES)

### INTRODUCTION

After seeing some of my preparations, Dr. H. E. Jordan suggested to me that a more extended study might add something to our knowledge of amitosis in the growth and repair of the living mechanism. Upon careful examination of the preparations of bone cells, many stages of nuclear and cells division were noted. These observations showed that amitosis was the only method of division present. Other preparations were made from various ages of bone and from various animals. In these preparations all stages of amitotic division were present, but not a single mitotic figure could be found. In the light of the recent discussions on the prevalency, cause, and significance of amitosis, these observations are very suggestive, especially since the various stages of division are so numerous and clear. Of greater import, however, for our knowledge of cell division is the observation that in the multiplication of bone cells two types of amitosis occur, and that these types are not only due to the difference in the form of the dividing nuclei, but that the form of division is also determined by the activity and position of the centrosomes.

### MATERIAL AND TECHNIQUE

The parietal bones of new born to three-weeks-old rats and the nasal bones, especially the ethmoid of the dog and rabbit, served as material for this study. Small pieces of bone are fixed in 95 per cent alcohol. The technique for their further preparation is the same as that described in my previous paper.

Staining with iron hematoxylin was attempted, but without success. It was hoped that a clearer picture of the centrosomes would be obtained by this method, but the process corroded and stained the bone to such an extent that the details were obscured. Centrosomes can be detected with considerable clearness in gentian-violet preparations, but they are not distinct in all cases.

Considerable difficulty was at first encountered in obtaining permanent mounts. Gentian violet has a tendency to fade if mounted in balsam. Quite satisfactory results are obtained, however, by mounting overstained preparations in balsam and exposing them to light for a month or more. In ordinary preparations the nuclear stain was well preserved, but the cytoplasmic coloration disappeared perceptibly. After some experimentation it was found that almost perfect preservation can be obtained in Canada balsam by observing the following precautions: 1) After dehydrating in absolute alcohol, wash in several changes of benzol to remove all traces of alcohol. 2) Mount in warm, thick, neutral balsam. I have several slides, prepared according to this method over a year ago, in which the staining is still almost perfect. It may be of interest to note that preparations can be preserved in benzol indefinitely, even in strong light, without fading.

#### DESCRIPTION

##### *Ratio of dividing to non-dividing cells*

In order to obtain a somewhat accurate idea of the ratio of dividing to non-dividing cells, it is necessary to consider both young and old bone. This study, however, yields only generalized results, since the ratio shows considerable range of variation in different bones and in different regions of the same bone. Thus in the ethmoid bone of an adult dog the greater number of cells show no signs of division; in some fields none, while in others as many as one cell out of every three are in some stage of division. In the parietal bone of a twenty-one-day rat a considerable range of cell age is encountered, and it shows that divid-

ing cells are more abundant in the younger than in the older area, yet in both regions the proportion is quite variable. This variability is also obvious in very young bone, although in it most cells are in some stage of proliferation (figs. 10 and 12). (See also figs. 2 and 6 of my previous article on bone cells.) Apparently this dividing process is a periodic affair, as shown by the fact that in a given region, especially in young bone, all the cells are practically in the same stage of proliferation. Because of this variability, the exact ratio of dividing to non-dividing cells cannot be given. It may be stated, however, that in young bone such as is found in one- to twenty-day-old rats, proliferating cells are so abundant that almost any stage of division can be found without difficulty. In older bone this occurs with less frequency.

#### *Nature of cell division*

In my previous paper it was shown that all cell division in bone is accomplished by amitosis. This requires little further description but can readily be noted in the accompanying figures (pl. 1). There is no sign of chromatin rearrangement nor of the disappearance of the nuclear wall in any case. At any stage the chromatin of the nucleus is grouped in small masses and irregularly distributed with a slight condensation at the periphery. This structure should be compared with the nucleus of a non-dividing cell as shown in figure 1.

#### *Types of amitosis*

It is a very striking phenomenon that among dividing bone cells two nuclear forms occur. In the one case the nucleus is dumb-bell shaped, while in the other it is horseshoe-like in form. Amitotic cells with the dumb-bell nuclei are not very common and usually occur in old bone, but may occasionally be seen in young bone also. Three stages of dumb-bell amitosis are shown in figures 2, 3, and 4. In figures 2 and 3 the two ends of the nucleus seem to be drawn in opposite directions, causing a rarefaction of the nucleoplasm in the middle which is followed by a constriction. The arrangement of the nuclear chromatin

is not different from that of the non-dividing nucleus as shown in figure 1. Figure 4 shows the nucleus completely divided. The two halves have moved in opposite directions and the cytoplasm at the plane of nuclear division is so constricted that the resemblance to true cell division is complete except for a narrow cytoplasmic connection. Whether such cytoplasmic connections always remain as narrow canalicular processes or sometimes divide completely to form two separate cells, I am unable to say. Certain it is that some cytoplasmic processes which are continuous with similar processes of sister cells are completely pinched off in the process of growth and become separated by bone matrix, but whether all of the processes are ever thus separated to form non-communicating cells, I am unable to say, since I have observed no such cases.

Another important structure, the centrosome, should be noted in connection with the dumb-bell-shaped nucleus. Centrosomes are not often clearly seen in dividing bone cells. This is especially true for this type of amitotic division because it is not frequently found. Figure 2 shows a cell in which the centrosomes are unmistakably distinct. They are placed in the cytoplasm at the two ends of the dumb-bell-shaped nucleus. The centriole within one centrosome is somewhat elongated, but the entire length was not visible in a single plane of focus. It appeared to lie at an angle of about  $45^\circ$  with this plane. Figure 2 shows the centriole as it appeared under a shifting focus. While in cells of this type centrosomes are not commonly seen, the centrosomes of this particular cell were so unmistakable, and duplicated so perfectly other less distinct observations, that it warrants the conclusion that this position of the centrosome is the rule for the dumb-bell type of amitosis.

By far the majority of the cells divide by the horseshoe type of amitosis. In young bone almost any stage of this type of cell division can be found, and one may often find every cell in some stage of division. It usually happens, however, that in a given field or even in a given parietal bone all dividing cells have approximately the same nuclear form. The various stages in the horseshoe type of amitosis are shown in figures 4 to 12.

Clear pictures of centrosomes are fairly common, but in this connection it should be remembered that a very large number of this type of cells are available from which to pick good stages. That many of the cells do not show centrosomes clearly can be explained by the fact that they may be obscured by other bone cells lying at a higher or lower level; or the cell may be so oriented that its own nucleus is the obscuring factor; or again in many cases protoplasmic processes viewed on end appear as deeply stained dots easily confused with granules and often obscuring the centrosome; or, finally, indistinct vacuoles lying in the granular cytoplasm appear so much like centrosomes that the picture is confusing. Thus in many cells the centrosomes are present, but cannot be cited as typical because of these obscuring structures. The various possibilities of the horseshoe type of amitosis are shown in figures 5 to 12. Figure 5 is a typical example of a cell in the early stages. At first the nucleus is kidney shaped with the centrosomes in the position of the hilum. The bending of the nucleus then increases so as to produce the horseshoe shape. The centrosomes do not migrate apart as they do in the dumb-bell-shaped nucleus, but remain in the concavity. It seems that the nucleus changes in such a way that each part of it remains as near the centrosome as possible. Often the two ends of the nucleus encircle the centrosomes to such an extent that they touch each other or even overlap. In this case, when viewed from the side the nucleus is ring-shaped with the centrosomes enclosed within.

A dividing nucleus is shown in figure 6. Division here is different from that in the dumb-bell type. Instead of becoming elongated, the nucleus is pinched in two without any perceptible moving apart of the daughter nuclei. The resulting kidney-shaped nuclei then separate a little (fig. 7) and the cell engages in cytoplasmic constriction (fig. 8). Later stages in cytoplasmic constriction are seen in figures 10 and 12.

Nuclear division does not always result in only two nuclei, but nucleic horseshoes sometimes split into three, four, or more daughter nuclei (figs. 9 and 11). Multinuclear cells are not numerous and are found in young bone. Whether such cells

really undergo cytoplasmic division cannot be stated with certainty, but pictures like figures 10 and 12 afford strong evidence that they do. That the cells in figure 10 are the product of a trinuclear cell can hardly be questioned. The cells in figure 12 may have resulted from two mother cells, but it is highly probable that they are the product of a tetranucleated mother cell.

The centrosomes in these multinucleated cells always lie in the center of the ring of nuclei. In such cells a variable number of centrioles are present. The number of these usually corresponds to the number of nuclei, but sometimes the number is considerably larger. This structure seems to correspond to the pluricorpuscular centrosomes which Jordan observed in polykaryocytes.

It is a striking fact that in polynuclear cells the cytoplasm is very abundant (figs. 9 and 11). It may be that this large amount of cytoplasm is a factor which determines the number of daughter nuclei into which a given nucleus shall divide.

Another structure which is common is the non-nucleated mass of protoplasm almost completely separated from the cell. Two such masses are shown in figure 2 and smaller masses in figures 7, 8, and 9. In rare cases small bits of nucleoplasm are included therein, as was indicated in my previous paper. Such structures are indicative of cytoplasmic separation without nuclear involvement.

#### SIGNIFICANCE OF AMITOSIS

The place of amitosis in the vital mechanism is a question which has been under critical discussion since Strasburger ('82) and Waldeyer ('88) advanced the view that amitosis in vertebrates was a survival of a primitive process. Since then many theories have been presented, some of which consider amitosis as a degenerative process or the end-stage in the series of cell divisions. Within recent years considerable evidence has been presented which indicates that it may be a normal generative process.

The following tabulation of the literature indicates the adherents to the various theories advanced.

1. Amitosis occurs in highly specialized cells and is followed by degeneration:

- Chun ('90) cited by Ziegler.  
 Flemming ('91, '92). In wandering cells which are on road to ruin.  
 Von Rath ('91, '93). In spermatozoa.  
 Ziegler ('91). In intense secretory cells.  
 Toyoma ('94). In the testes of the silkworm.  
 Krompecher ('95). In bone-marrow.  
 Wilcox ('95). In giant spermatozoa "really come to naught."  
 Plate ('98). In dying cells of tracheal epithelium of Janelles.  
 deBruyne ('99). In ovarian follicle of Hemiptera and Orthoptera.  
 Wilson ('00). A secondary process in most cases.  
 Gross ('01). In germ cells of thirteen species of Hemiptera.  
 Payne ('12). In *Gelastocoris*, the cells which apparently multiply by amitosis do not produce ova.

2. Generative amitosis does occur and it is a normal method:

- Arnold ('83-'84) Bone-marrow cells and leucocytes.  
 Löwit ('91) In leucocytes.  
 Verson ('91) In blind end of testicular follicle of *Bombyx*.  
 Frenzel ('91) In intestinal glands of crayfish.  
 Paladino ('93, '95) The new formations of the placenta in mammals.  
 Reguard ('00) In Sertoli cells and spermatogonia of rats.  
 Camaniti ('03). In liver cells (as important as mitosis).  
 Hargitt ('03). In hydroids.  
 Klemensiewicz ('03, '04.) In blood cells (as important as mitosis).  
 Child ('04). In the developing tissues of *Moniezia*.  
 Gurwitsch ('04).  
 Maximow ('08). Normal in mesenchyme cells of young dog embryo.  
 Glasser ('08). In entoderm of *Fasciolaria* (during development he found mitosis in 13 per cent and amitosis 87 per cent).  
 Rubaschkin ('08). In mesenchyme of embryo in region where gonads later form.  
 Foot and Strobell ('11). In ovaries of *Protenor*. Cells become ova.  
 Descilleuls ('14). In epithelial cells of viterine cornea.  
 Arber ('14). In cortical cells of root of *Stratiotes*.  
 McLean, ('14). In cortical parenchyma of aquatic angiosperms.  
 Jordan ('13). In ciliated cells.

3. Cells may divide by amitosis due to external conditions and later divide by mitosis again:

- Meves ('91, '94, '96). In spermatogonia of salamandra. Due to cold.  
 Nathanson ('00). In plants external condition ether).  
 Gerasimoff ('92). In *Spirogyra* (says nothing of their return to mitosis).



- Preusse ('85). Ogonia of Hemiptera.  
 Pfeffer ('99). In Spirogyra.  
 McGregor ('99). In Amphiuma.  
 Wasielewski ('03, '04). In root tip of Vicia.  
 Patterson ('08). In pigeon's blastoderm.  
 Wieman ('10). In ovaries and testes of Leptinotarsa.  
 Jordan ('13). In onion root.

4. Occurs in regions of rapid growth where the demand for certain foods exceeds the supply. The nucleus in such a condition is not in the proper equilibrium to divide by mitosis and so resorts to the easier method of amitosis.

- Osborn ('04). In food ova of Fasciolaria.  
 Child ('07). In both invertebrates and vertebrates.  
 Patterson ('08). In pigeon's blastoderm (due to special physiological conditions.)  
 Wieman ('10). In reproductive cells of Leptinotarsa (change in nutritive supply).  
 Glasser ('05). A demand for a greater nuclear surface.  
 Jordan ('19). In blood and bone-marrow cells of frog. Lack of nutritive supply due to high degree of specialisation.

5. Amitosis is a purely pathological phenomenon:

- Glasser ('07). Food ova of Fasciolaria.  
 Pacaut ('09). In epithelial cells of mammalian cornea.

6. Amitosis is due to external mechanical pressure:

- Nowikoff ('08, '09, '10). In cartilage, sinew, and bone cells of the mouse embryo.

7. Amitosis is a nuclear matter only. The cytoplasm does not divide, thus giving rise to a multinuclear cell:

- Heidenhain ('94).  
 Karpow ('04). Leucocytes an exception. (His work was on the epidermis of amphibians and epithelium of the urinary bladder of mammals.)  
 Schurhoff ('15). In Ranunculus.  
 Macklin ('16). In the chick embryo.

8. Amitosis is a normal method of division in vertebrates only:

Nakahara ('18) makes the following statement: "Amitosis . . . is not a method of cell multiplication, nor a sign of degeneration or senescence of cells, but, whenever it occurs, it seems to indicate an intense activity in the vegetative functions of the cell."

Besides the views expressed above there are those who claim that amitosis as observed in the above cases is not amitosis at all. They would call it pseudo-amitosis or simply a peculiar type of mitosis. We shall mention only a few of those who so discredit amitosis:

- Hacker ('00). In eggs of cyclops.
- His ('00). In roots of *Listera* and *Orchis*.
- Hargitt ('06). In clava *leptostyla*.
- Richards ('09). In oögenesis of *Taenia*.
- Harman, Mary T. ('13). In sex-cells of *Taenia teniaeformis*.
- Conklin ('17).

#### DISCUSSION

The present observations on bone cells throw no further light on theories 3 and 8, while theories 1, 5, and 6 are negatively answered. The first part of theory 1, however, must receive some attention. Bone cells are highly specialized cells, and it is probable that this specialization does influence amitosis. That these cells have entered a stage of degeneration is not supported by these studies. Macewen believes that bone cells may at any time give rise to osteoblasts, thus attributing to them a generative or regenerative importance.

Theory 6, which states that amitosis is a mechanical pinching in two of the cells due to external pressure, was formulated by Nowikoff as a result of his observations on amitosis in cartilage, tendon, and bone. His two pictures of bone cells in his 1910 article on "Zur Frage nach der Bedeutung der Amitose" indeed appear to support his view. In my own preparations no such pictures were found. The only examples from which such a view could be forced are shown in figures 2, 3, and 4. In figures 2 and 3 there is only a slight rarefaction of cytoplasm, while the nucleus is distinctly constricted but without signs of external constriction. In figure 4 the cytoplasm is constricted, but the nucleus is completely divided into two daughter nuclei. These observations give the impression that cytoplasmic constriction occurs as a normal process following nuclear division. In dividing cells in which the horseshoe-shaped nucleus occurs—which

is by far the most common method, there is not the least indication of external pressure. The only place where this theory might apply is in the case of protoplasmic fragmentation; that is, protoplasmic masses, with or without small nuclear remains, isolated from the main cell and connected with it by only a narrow cytoplasmic process (figs. 2 and 8 and fig. 4 previous paper). In such cases the deposition of bone takes place more rapidly on certain surfaces of the cell than on other, thus causing the constriction.

In reference to the other theories, it should be said that bone cells always multiply by amitosis, and therefore these observations support theory 2. Theory 4 also finds support in these observations in that amitosis occurs in young and rapidly growing bone. The growth is so rapid that the 4-mm. parietal bone of a newborn rat is 9 mm. in diameter at the age of fourteen days. In such rapidly growing tissue it is reasonable that the question of food supply may have considerable influence. Furthermore, in bone the individual cells are connected with the source of food supply by only narrow cytoplasmic processes, which channels may be insufficient to supply the entire cell. While such views seem reasonable, they by no means are final, since any statement regarding function will of necessity be theoretical, and the study of bone cells warrants no positive statement regarding the physiological reason for amitotic cell division.

In mitosis there is a mechanism, the centrosome, which is apparently directly concerned in cell division. Some would have this mechanism an indispensable factor in all cell division. They regard all other signs of cleavage as incomplete division. Thus, theory 7 admits that the nucleus may divide by amitosis, but that no cytoplasmic division occurs in such cases. The division of leucocytes however, has been suggested, by adherents, of this view, as an exception to the rule. On the other hand, there are those who hold that complete cell division does occur in amitosis. Jordan, in his paper on "Amitosis in the epididymis of the mouse," has shown that cytoplasmic division follows amitotic division of the nucleus. Those who have described amitosis for sex cells believe in cytoplasmic division after nuclear division.

In unpublished observations on red blood cells of *Necturus*, Dr. W. S. Miller, of the University of Wisconsin, has found that these blood cells divide amitotically. This division concerns not only the nucleus, but the cytoplasm as well, thus giving rise to two distinct cells. Doctor Miller has been kind enough to allow me to study this material and verify his findings.

In the face of these findings, it is impossible to say that amitosis is a nuclear matter only. The observations on bone cells do not help us in solving this question. Since bone cells form a syncytium, the cytoplasm does not completely separate in the dividing cell. In reality the cytoplasm is continuous, but the constriction is so nearly complete that the cells are regarded as units. Those who believe that amitosis results in incomplete division will find support in the division of bone cells. It seems, however, that a tissue whose cells form a syncytium is not a fitting tissue on which to determine this phase of the question.

Leaving out all theoretical possibilities which these observations suggest, we can make the following assertions:

1. Amitosis occurs normally among bone cells.
2. Amitosis occurs in rapidly growing bone.
3. Bone cells in which amitosis occurs are specialized cells.
4. Bone cells apparently do not divide completely, but form a syncytium.

#### TYPES OF AMITOSIS AND THE RÔLE OF THE CENTROSOMES

M. Nowikoff says, "Some authors differentiate two types of amitosis. The first begins with an elongated nucleus, which later takes on a biscuit like form. The bridge between the two enlarged nuclear ends becomes finer and finer and finally tears completely. Amitosis of the second type results from the formation of a notch in the nuclear membrane, which becomes deeper and deeper and in this way pinches the nucleus into two halves." Wasielewski recognizes these two types.

The description of the first agrees with the dumb-bell type described above. Nowikoff claims that it is the only type found in bone and tendon cells. This does not correspond at all with my observations on bone, which show that by far the majority

of the dividing nuclei have a horseshoe-shaped nucleus. Many of the cells which Nowikoff illustrates and which he classifies as cells of the first type really appear to belong to the horseshoe type. Nowikoff studied sections of bone, and that may have led to a misinterpretation of the shape of the nucleus. For example, a horseshoe-shaped nucleus when cut at right angles to the bars of the horseshoe, near or at the bend, appears dumb-bell shaped. Or, if it is cut at an angle of  $45^\circ$  with the bars of the horseshoe, it will look like a slightly bent dumb-bell, as many of his illustrations show. Thus the many different forms depend on the plane of section. In cartilage he found so many different forms representing all stages between the first and second type that he concluded that no two distinct types existed.

This question, however, deserves a little further consideration. The dumb-bell and horseshoe types in bone are too distinct and constant to pass unnoticed. This distinction becomes all the more apparent when we note that the shape of the nucleus is definitely related to the position of the centrosomes.

In the literature on amitosis very little is said regarding the centrosome. Many believe that it is entirely absent in cells that divide by amitosis. Among them are Hennequy, Lenhossek, Zimmerman, Heidenhain, Fuchs, Joseph, and Jordan in ciliated cells, and others. Among those who have observed centrosomes in amitotically dividing cells are Meyes, Studnicka, Fischel, Eismond, Henry, Benda, Gurwitsch, Ach, Wallengren, Ikeda, Erhard, Maximow, Nowikoff, Saguchi, Jordan, and others.

The above list indicates that the centrosomes have been quite generally found in amitotically dividing cells. Whether these centrosomes are of any functional importance in amitosis is a question which is usually negatively regarded. Flemming and Maximow always find the centrosome close to the nuclear membrane and at the place where the constriction takes place. In his 1910 paper Nowikoff tries to support Maximow's view regarding the centrosomes. However, in figure 45 of his 1908 article he shows two centrosomes which lie one at each of the two enlarged ends of the dumb-bell-shaped nucleus. In that case the centrosomes certainly have moved apart just as they do

in case of mitosis. Saguchi presents a similar moving apart of the centrosomes in his figure 49, plate 3. In this case the nucleus has not moved apart as far as the one shown by Nowikoff or the one in my figure 2. This incomplete polar movement of the centrosomes accounts for the shape of the nucleus.

In my observations on bone cells a definite relationship between position of the centrosomes and the shape of the nucleus could be established. In trying to correlate this observation with those of other observers and with the various theories on amitosis, it appeared that amitotic nuclear forms can be classified according to the functioning ability of the centrosomes. If it is true, as many believe, that a cell may divide amitotically and then return to the mitotic type of division, and if the centrosome is active in mitosis and inactive in amitosis, then it is not strange that intermediate stages of centrosomal activity should occur. Food conditions, specialization, or external stimuli may disturb the equilibrium of the nuclear chromatin while the centrosome remains functional, thus causing amitotic division, or these disturbing factors may impair the migrating power of the centrosomes; or finally they may paralyze the entire mechanism which is active in mitosis and thus cause amitotic division.

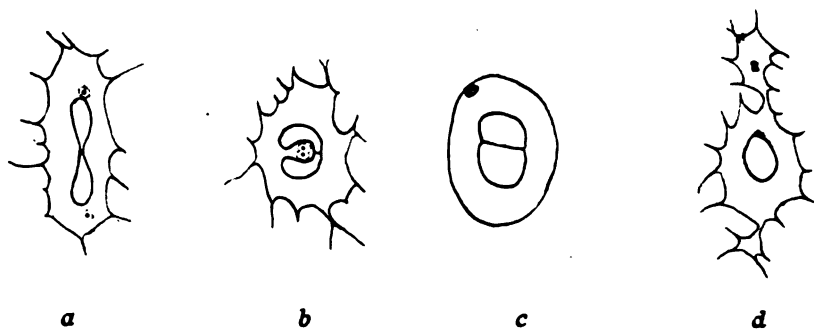
In view of these observations and considerations, the following classification of amitotic types seems warranted:

Type I. The nucleus is dumb-bell shaped. The centrosomes have moved apart and placed themselves one at each end of the elongated nucleus and apparently 'exert a pull on it' (text fig. 1 a).

The only difference between this type of amitosis and mitosis is the inability of the nuclear chromatin to rearrange itself. Examples of this type are shown in figures 2, 3, and 4. The illustrations of Nowikoff and Saguchi referred to above belong under this type.

Type II. The nucleus is horseshoe shaped. The centrosomes do not move apart, but they still 'exert a pull' on the nucleus. In some cases the movement of the nucleus around the centrosomes progresses to such an extent that the ends of the nucleus meet, thus forming a ring around the centrosomes (text figure 1, + b).

This type is well represented in bone cells by figures 5 to 12. Jordan describes this type in the blood and the bone-marrow of the frog and in the bone-marrow of the rabbit. Flemming's figure 5 is of this type. Most of Nowikoff's and Maximow's figures look as though they belonged to this type. Thus Maximow's figure 9 shows a nucleus which looks dumb-bell-like in shape. A comparison of this nucleus with the middle nucleus of my figure 10, which is a horseshoe-shaped nucleus viewed from one end, makes the similarity very apparent. Some of Arnold's



Text fig. 1 Diagrammatic sketches representing the four types of amitotic nuclear division. a, Type 1. The nucleus is dumbbell-shaped with the centrosomes at the poles of the dividing nucleus. b, Type 2. The nucleus is horseshoe-shaped with the centrosomes within the bend of the nucleus. c, Type 3. A plate like structure marks the plane of division of the oval-shaped nucleus. Centrosomes are near the surface of the cells, but they may be found in other positions or may be entirely absent. d, Cell fragmentation. The mass of protoplasm on top contains a small amount of unorganized nucleoplasm. The mass at the bottom contains no nuclear material.

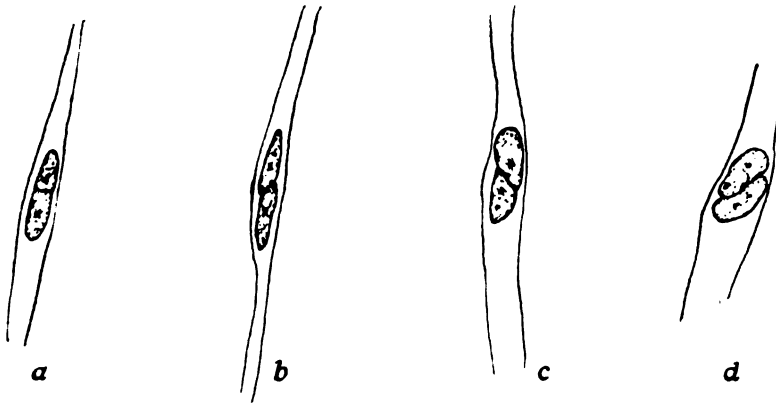
observations belong here. Heidenhain has given the most extended list of descriptions and illustrations of this type.

Type III. In this type the centrosome is functionless; it neither moves apart nor exerts any influence on the nucleus. In some cases the centrosomes may be absent. In the early stages of division a plate-like structure marks the plane of cleavage (text fig. 1, c). A narrow notch on one surface of the nucleus often initiates separation of the daughter nuclei. The ciliated cells illustrated by Jordan and Saguhei and the fat cell by Nakahara belong to this type. I have found many nuclei

of this type in smooth muscle cells of the human uterus (text fig. 2, a, b, c, and d). Such cases are also quite common among lymphocytes.<sup>1</sup>

#### CELL FRAGMENTATION

There are cases of unequal division which in reality are not true cell division, but only types of cell fragmentation (text fig. 1, d). Nowikoff found such cases in tendon and bone cells. He decided that such division was due to external pressure.



Text fig. 2 Camera-lucida drawings of smooth muscle cells from a pregnant human uterus. a, b, c and d show different forms of the third type of amitotic division. Original drawings were made on the scale of  $\times 1200$ , but were reduced in reproduction to  $\frac{1}{2}$  of the original.

Maximow found them in mesenchymal tissue. The large masses of bone-cell protoplasm with or without nuclear fragments which I have found in bone (fig. 2, also fig. 4 of previous paper) belong to this type.

<sup>1</sup>Dr. C. H. Bunting, of the Pathological Laboratory of the University of Wisconsin, called my attention to this type of division among lymphocytes.



## SUMMARY

1. Bone cells divide by amitosis.
2. Division is more rapid in very young than in older bone.
3. Cell division seems to be a periodic process. In a given area all of the cells are in practically the same stage of division.
4. Amitosis is the normal method of bone-cell multiplication. That amitosis occurs rather than mitosis *may* be due to one or all of the following factors. a) High degree of specialization of cells concerned. b) Disturbance of nutritive equilibrium. c) Insufficient nuclear surface for the proper metabolic functioning of the cell.

5. Three types of amitosis are present in bone-cell division, and they are determined by the activity and position of the centrosomes. In one type the nucleus is dumb-bell shaped and the centrosomes are placed at the opposite poles of the nucleus. In the second type the centrosomes are placed in the center of the horseshoe-shaped nucleus. The latter type can hardly be considered as amitosis, but should be regarded as cell fragmentation. In this latter case division seems to be entirely due to external pressure.

6. A correlation of the various accounts of amitosis shows that one other type of amitosis exists. In this type the centrosomes are in no way concerned in the process of division. Centrosomes, as such, may or may not be present. A plate-like structure indicates the plane of division.

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For a more extended bibliography on amitosis see Saguchi and Nakahara, referred to above.

## PLATE 1

## EXPLANATION OF FIGURES

All of these drawings are camera-lucida drawings of cells from the entire parietal bones of young rats, except figures 3, 4, and 5, which were taken from the ethmoid bone of a dog. These bones were fixed in 95 per cent alcohol and stained in gentian violet. The original magnification as indicated for each figure was reduced  $\frac{1}{2}$  in reproduction.

1 Normal bone cell from fifteen day-old rat. The nucleus is oval in shape.  $\times 1200$ .

2 Cell in early stage of division. Nucleus dumbbell shaped. Centrosomes at opposite poles of nucleus. Two large masses of protoplasm almost separated from main cell.  $\times 1200$ .

3 Cell from ethmoid bone of dog. A little later stage of nuclear division than figure 2. Cytoplasm is not constricted.  $\times 1450$ .

4 Cell from ethmoid bone of dog. Nucleus completely divided. Cytoplasm constricted. Centrosomes are faintly seen, but the centrioles are not visible.  $\times 1450$ .

5 Cell from the ethmoid bone of a dog. Early stage in the horseshoe type of amitosis. The centrosomes are placed at the center of the horseshoe-shaped nucleus.  $\times 1450$ .

6 The horseshoe-shaped nucleus has just divided. Centrosomes at the center.  $\times 1600$ .

7 A later stage in the division of a horseshoe-shaped nucleus. The two daughter nuclei are kidney shaped and only slightly separated. The centrosomes are four in number and still centrally placed.  $\times 1600$ .

8 Late stage in the horseshoe type of amitosis. The cytoplasm is deeply constricted. The irregular shaped nuclei are due to improper fixation.  $\times 1450$ .

9 These three nuclei are the product of the division of a horseshoe-shaped nucleus. Centrosomes centrally placed.  $\times 1600$ .

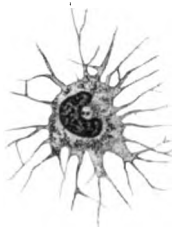
10 Three cells derived from a cell with a horseshoe-shaped nucleus. The nuclei of these cells are already in the early stage of division.  $\times 1450$ .

11 Pentanucleated cell with the nuclei still arranged in the shape of a horseshoe. Multicorpuscular centrosome within ring of nuclei.  $\times 1600$ .

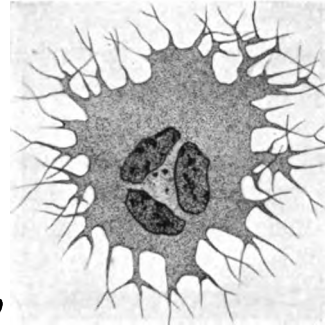
12 Four cells which are probably the product of a tetranucleated cell. Note the large protoplasmic connections.  $\times 1600$ .



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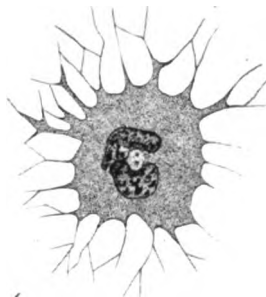
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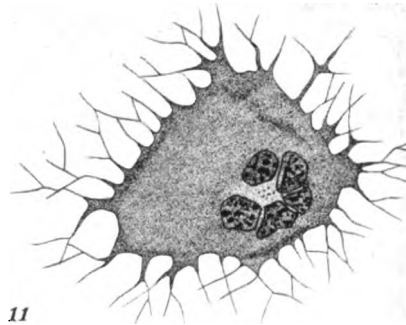
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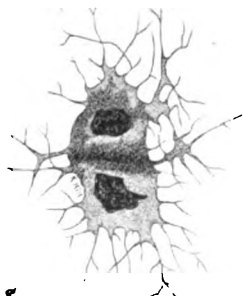
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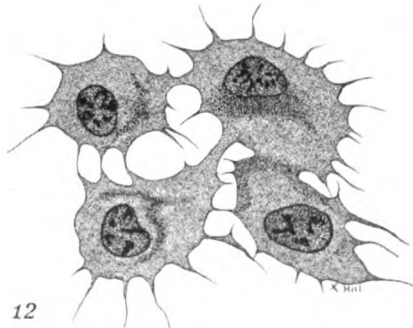
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12

Resumen por el autor, Eben J. Carey.

Estudios sobre la dinámica de la histogénesis. La tensión del crecimiento diferencial como estímulo para la miogénesis.

VII. La transformación experimental del músculo liso de la vejiga del perro, histológicamente en músculo estriado, y fisiológicamente en un órgano que manifiesta ritmicidad.

La diferencia esencial entre el músculo liso pálido de la vejiga y el músculo rojo estriado involuntario del corazón depende de la intensidad diferencial de la presión hidrodinámica a que han estado sometidas las células mesenquimatosas vesiculares y cardíacas. Variando la velocidad de la aplicación y la intensidad de la presión intravesical (que produce la tensión del músculo liso de la vejiga), durante un periodo de ocho semanas hasta alcanzar un grado comparable al que se encuentra en el corazón, el músculo no estriado vesical se transforma, histológicamente en músculo estriado y fisiológicamente en un órgano que manifiesta ritmicidad mientras se aplique el estímulo de la presión hidrodinámica. Desde el punto de vista dinámico o embriológico-funcional los diversos músculos, liso, cardíaco y esquelético, representan diferencias en la cantidad de trabajo que sobre ellos han ejercido las partes del embrión en vías de crecimiento diferencial durante los periodos de crecimiento activo. La diferencia esencial, fisiológicamente entre los diversos músculos, es pues su capacidad para trabajar, que a su vez depende de la cantidad de trabajo que ha sido consumida en su producción. La razón de los diferentes grados de energía que poseen los tipos de músculos es puramente un problema embriológico biomecánico y corresponde a la cantidad diferencial de tensión óptima que han experimentado estos músculos durante su periodo formativo, a causa de la existencia de una zona energética dominante extrínseca a la región de la miogénesis. Las pruebas obtenidas de estos experimentos, justifican la conclusión de que en lo referente al músculo estriado, la función determina la estructura en vez de lo contrario.

Translation by José F. Nonides  
Cornell Medical College, New York

## STUDIES IN THE DYNAMICS OF HISTOGENESIS. TENSION OF DIFFERENTIAL GROWTH AS A STIMULUS TO MYOGENESIS<sup>1</sup>

### VII. THE EXPERIMENTAL TRANSFORMATION OF THE SMOOTH BLADDER MUSCLE OF THE DOG, HISTOLOGICALLY INTO CROSS-STRIATED MUSCLE AND PHYSIOLOGICALLY INTO AN ORGAN MANIFESTING RHYTHMICALITY

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TWENTY FIGURES

#### INTRODUCTION

It has been the problem of the writer for a number of years to determine experimentally whether an undifferentiated mesenchymal cell subjected to an optimum tension would develop into a muscle cell. The technical details of this problem have not yet been surmounted. If, however, the various types of muscle cells found in the body, viz., smooth, cardiac, and skeletal, represent resultants of different degrees of optimum tension (Carey, '19-20 a; '20 b), then the proof for this idea would be forthcoming if non-striated muscle could be converted into cross-striated muscle by varying the velocity of application and the intensity of the tensional stimulus to a higher optimum degree.

It is held at the present time that the essential difference between the types of developed muscle is the presence or absence of cross-striations. From the purely static and structural standpoint, these cross-bars are the outstanding feature. On the other hand, from the dynamic or functional and embryological

<sup>1</sup> This thesis was granted first prize in the competition for the medical prize scholarship for original research by the medical faculty of the University of Chicago, Rush Medical College, 1921.

view, these muscle types represent differences in the amount of work that has been done upon them by the differential growing parts of the embryo during the active period of growth (Carey, '21). The essential difference, then, physiologically between the various muscles is their capacity for work, which in turn is dependent upon the amount of work that has been expended in their production. The reason for the different degrees of energy possessed by the types of muscles is purely an embryological biomechanical problem and corresponds to the differential amount of tensile work that has been expended in their formation by a dominant energetic zone extrinsic to the region of myogenesis.

This idea may be exemplified by comparing the heart and the bladder. The rotating blood stream winds up the embryonic cardiac mesenchyme into a spiral musculature for action by the tensional interaction of differential growth, like the key that winds up the spiral springs of an eight-day clock for movement. The tensional stresses exerted by the helicoidal circulation of the blood stream upon the cardiac mesenchyme is the dynamic stimulus for heart-muscle development. The blood gradually collects in the living chick embryo in the region occupied by undifferentiated mesenchyme, which ultimately becomes modified into cross-striated muscle. This modification or transformation is a gradual one. At the thirtieth hour of incubation the primitive mesenchyme begins to rhythmically pulsate, at first slowly, then more and more rapidly as growth continues and as the vascular channels become hollowed out and continuous. This is concomitant with a constantly greater increase in the volume of the blood. Huntington ('11), McClure ('15), Schulte ('14), and others have proved that the vascular channels are formed by the confluence of isolated vesicles due to blood pressure. The rate of the heart beat increases from 10 to 15 at the thirtieth hour to 150 to 190 per minute at the ninetieth hour. From the fortieth until the one hundred twenty-fifth hour the heart is composed of cells not unlike smooth muscle. But at about the one hundred twentieth-fifth hour cross-striations appear. Why?

On the other hand, the slowly excreted urine which collects in the bladder is the hydrodynamic factor causing the optimum tension for the smooth-muscle differentiation in the vesicular mesenchyme. The volume of the excreted urine that collects in the bladder per unit of time never reaches that of the blood that circulates through the heart in a corresponding temporal interval. Therefore, it is immediately evident that a greater amount of work is expended by the rapidly flowing blood stream which whorls the cardiac mesenchyme into striated muscle than by the excreted urine which causes a gradual tension in the vesicular mesenchyme leading to smooth-muscle formation as a mesenchymal reaction.

The essential difference, then, between the pale smooth muscle of the bladder and the red involuntary striated muscle of the heart is finally dependent upon the differential intensity of hydrodynamic tensional stimuli (work) to which the vesicular and cardiac mesenchymal cells are subjected, respectively. If the growing vesicular smooth muscle is subjected to a stimulus comparable to that found in the heart, will cross-striated muscle be differentiated? The answer is emphatically yes! The following experimental evidence puts at rest any further thought that in the embryo some intracellular hypothetical structural precursor within so-called myoblasts accounts for muscle origin as stated by the meaningless verbalism self-differentiation, when applied to myogenesis.

#### MATERIALS AND METHODS

A young female shepherd dog four weeks old was selected for the experiment. A suprapubic vesicular silver drainage tube, designed by the writer, of the type shown in figures 1, 2, 3, and 4, was transfixed in the bladder, March 30, 1921. This was accomplished with silkwork-gut ligature which was passed through the perforations in the disc of the tube and tied to exterior of the abdominal wall. The bladder of this pup presented the pale appearance of smooth muscle on inspection at the initial insertion of the drainage tube. Very little resistance to incision with the scalpel was presented by the bladder musculature. The vesicular wall measured 0.5 mm. in thickness. The bladder of a control



puppy four weeks old weighed 0.75 gram. At the first operation the bladder was examined histologically; it presented the appearance of typical smooth muscle and possessed transitional epithelium (figs. 9, 10, 11, 12, and 13).

Concentrated boric acid at 37.5°C. was passed through the bladder under varying intensities of pressure and at various intervals of time daily. The experiment is illustrated in the outline drawing (fig. 7). On May 21st, a second operation was performed for the purpose of excising a portion of the bladder for histological purposes and, if cross-striated muscle were found, to allow the musculature to revert to smooth muscle. The bladder muscle presented a deep red appearance and the resistance to incision with the scalpel had greatly increased. The bladder wall was 5 mm. in width, whereas the bladder in the control puppy was 1.9 mm. The bladder of a control puppy weighed 1.59 grams, whereas the bladder in the operated dog weighed 4.75 grams.

The insertion of the suprapubic tube was done for me by Dr. B. F. McGrath, Director of the Department of Clinical Laboratories. The steps in the operation were as follows:

- A. Strict asepsis was rigidly adhered to.
- B. Anesthetic: Ether, drop method.
  1. Bladder exposed by a suprapubic incision in the midline.
  2. Purse-string suture of chromicized catgut inserted high in anterior wall of bladder.
  3. Small opening in center of the circle formed by the purse-string suture.
  4. Three fixing sutures of silkworm-gut, each made as follows: From without, through *a*) all layers of the abdominal wall, *b*) anterior wall of bladder, and *c*) a perforation in the flange of the tube; from within, returning through *a*) a perforation in the flange of the tube, *b*) anterior wall of bladder, and *c*) all layers of the abdominal wall.
  5. Opening in the bladder wall stretched and the flange of the tube inserted into cavity of bladder.
  6. Purse-string suture tightened about tube and ends tied.
  7. Abdominal wall wound closed, up to tube, with interrupted silkworm-gut suture.

8. The silkworm-gut loops, for fixing the flange of the tube in place, tightened and the ends tied.

#### EXPERIMENTAL RESULTS

The volume of boric acid passed through the bladder was gradually increased from April 3rd, five days after the suprapubic drainage tube had been inserted in the bladder, until May 21st (table 1). The volume of the fluid was regulated by means of a screw-clamp, and as soon as the puppy showed signs of distress due to vesicular distention the circulation was reduced or stopped completely until the fluid was passed by the urethra. On April 3rd the experiment lasted for one-half hour, during which time 20 cc. of fluid had passed through the bladder and out through the urethra. The urethral passage of urine was of the nature of clonic, short, rapidly recurring contractions. One clonic spasm occurred every two minutes and each spasm consisted of about ten short contractions.

As time went on the clonic nature of the bladder contractions was lost and a greater volume of fluid was passed with each urethral relaxation. Corresponding to each individual contraction of the bladder, a complete urethral relaxation occurred. The resemblance of the full contractions of the bladder to that of the heart and the perfect coordination of urethral relaxation and subsequent closure like a cardiac valve gave an ideal experimental condition for inducing in the bladder the circulatory changes found in the heart. A mechanical valve was occasionally used between the elevated pressure reservoir and the mercury manometer. This device gave a still further resemblance to the nature of the vesicular contractions, under a hydrodynamic stimulus, comparable to that found in the heart.

The mercury manometer, unfortunately, was not used throughout the experiment. The striking results obtained later suggested the use of this instrument in order to record more accurately the vesicular contractions, their variation under varying pressure and volume conditions, and their absolute inhibition by withdrawing the stimulus by closing the screw clamp and stopping the circulation.

TABLE 1

DATE OF OPERATION	LENGTH OF EXPERIMENT IN HOURS	VOLUME OF BORIC ACID PUMPED THROUGH THE URETHRA IN CUBIC CENTIMETERS	BEATS PER MINUTE, AVERAGE	WEIGHT OF DOG IN GRAMS	REMARKS
March					
30				2,400	
31				2,310	
April					
1				2,390	
2				2,415	
3	0.5	20	10	2,500	Dog experiences distress due to vesicular tension
4	0.5	25	10	2,575	
5	0.5	20	10	2,600	
6	1.0	35	14	2,690	
7	1.0	30	12	2,740	
8	1.0	40	14	2,770	
9	1.0	55	15	2,830	
10	1.5	65	20	2,850	
11	1.5	78	20	2,890	
12	2.0	100	21	2,970	
13	2.0	110	25	3,020	
14	2.0	130	24	3,110	
15	2.0	150	30	3,200	
16	2.0	190	32	3,220	
17	2.0	240	35	3,225	Slight distress
18	2.5	250	40	3,225	
19	2.5	275	38	3,225	
20	2.5	275	40	3,230	Normal excretion of urine
21	2.5	300	49	3,250	
22	2.5	335	50	3,267	
23	2.5	410	50	3,270	
24	2.5	500	54	3,175	
25	3.0	525	57	3,285	Greatest weight
26	3.0	600	60	3,185	
27	3.0	650	60	3,215	
28	3.0	700	65	3,230	
29	3.0	700	60	3,120	
30	3.0	800	70	3,211	
May					
1	3.5	1,000	70-100	3,223	No distress, even sleeps during experiment
2	3.5	1,250	70-100	3,155	
3	4.0	2,000	70-150	3,115	
4	4.0	2,500	70-200	3,160	
5	4.0	3,900	70-200	3,110	
6	5.0	3,500	70-200	2,930	Dog lost appetite, drinks only water, becoming emaciated
7	5.0	4,800	70-250	2,825	
8	5.0	5,000	70-250	2,800	
9	5.0	5,250	70-300	2,800	

TABLE 1—*Continued*

DATE OF OPERATION	LENGTH OF EXPERIMENT IN HOURS	VOLUME OF BORIC ACID PUMPED THROUGH THE URETHRA IN CUBIC CENTIMETERS	BEATS PER MINUTE, AVERAGE	WEIGHT OF DOG IN GRAMS	REMARKS
10	5.5	6,900	70-300	2,790	
11	5.5	8,500	70-300	2,750	
12	6.0	10,000	70-300	2,780	
13	6.0	15,000	70-300	2,760	Forced feeding
14	8.0	18,000	70-300	2,790	
15	8.0	25,000	70-300	2,740	
16	8.0	30,000	70-300	2,720	Very slight excretion of urine
17	8.0	30,000	70-300	2,700	
18	8.0	35,000	70-300	2,700	
19	9.0	40,000	70-300	2,690	
20	9.0	45,000	70-300	2,650	
21	10.0	50,000	70-300	2,600	

The common cardiac conditions could be imitated at will. A bradycardia, normal cardiac regularity, tachycardia, extra systoles, group formation, pulsus alternans and ventricular fibrillations could be induced by varying the pressure and by either using or not the mechanical valve between the elevated pressure reservoir and the mercury manometer.

The bladder of this dog prior to the operation, March 29th, passed an average of 250 cc. of urine in twenty-four hours. On May 20-21, this same bladder passed the enormous volume of 50,000 cc. of boric acid during ten hours of experimental observation. This prolonged increase of vesicular pressure prevented the urine from being excreted and set up a uraemic toxæmia which so lowered the resistance of the young dog that it died twenty-four hours after the second operation. On postmortem examination both ureters were enormously dilated with urine and measured 10 mm. in diameter. The nephritic pelvis were distended with urine and both kidneys were markedly hydro-nephrotic. The writer intends to present a series of dogs and microscopic demonstrations at the next meeting of the American Association of Anatomists.

Figure 14 shows the manometric curves from the inlet tube of the bladder during the first hour and fifteen minutes of the

experiment May 21st, after the dog had had a night's rest. A constant volume and pressure were maintained throughout the observation. The varying irritability and response of the bladder during this observation are clearly shown. At the beginning, line 1, the bladder is not so irritable as subsequently, lines 2 to 5. At first the contractions are few in number, but as the bladder becomes more irritable to the constant hydrodynamic tensile stimulus it responds with more celerity.

By reducing the pressure the vesicular contractions are retarded (figs. 15 and 16, line 1), and by complete inhibition of the circulation, no contractions are elicited (fig. 16, lines 1, 2, 3, 4, 5, 6 and 7). The stimulus that causes the rhythmic beat of the bladder is hydrogenic in nature. The rhythmic beat is dependent also on the irritability of the responding mechanism. This fact is immediately evident by inspecting (fig. 14, lines 1, 2, 3, 4, and 5).

Acceleration of the vesicular beats may be induced by increasing the volume and pressure of the fluid flowing into the bladder. This is clearly shown in (fig. 20). By regulating the fluid pressure, a condition may be induced whereby the bladder responds vigorously and regularly. When the stimulus was so regulated that sixty-five to seventy-five beats per minute were established, the bladder reacted with regularity and absolutely no distress was experienced by the puppy. The dog could be maintained in a recumbent position for hours and would breathe regularly even sleep for half an hour or so while the bladder was pumping at the rate of seventy-five times per minutes. There were no conscious efforts on the part of the dog with these rhythmic contractions of the bladder once the experiment was under way and the vesicular irritability had been reestablished after the dog had had a night's rest. Figure 14 shows the bladder beating at the rate of about fifty per minute during one hour and fifteen minutes of observation; this record was made with a slow drum. The puppy slept practically throughout the time that the manometric curves were being recorded.

The structure of these pressure curves is definitely shown by figures 17 and 18 made on a fast drum. The up-stroke represents bladder distention with the concomitant increased back pressure

recorded through the inlet tube. The down-stroke represents vesicular contraction simultaneous with urethral relaxation corresponding to the time that the bladder is being emptied and the concomitant reduction of back pressure through the inlet tube. These curves were made with the mechanical valve working between the elevated pressure reservoir and the mercury manometer. They are of different types: some are single, others double hillocks, while some show the pressure rising gradually and then a sudden release with the contraction of the bladder. Certain curves show a comparatively acute summit, others a definite plateau. This indicates a variable irritability and response of the modified vesicular musculature to the constant extrinsic tensile stimulus.

The contractions of the bladder musculature are active and independent of the action of the heart and respiration (table 2). With each bladder contraction determined by the manometer and by palpation there is an expulsion of urine. This expulsion is due to vesicular contraction and not to a passive transmission through the bladder on account of any active zone extrinsic to the contracting vesicle. The abdominal musculature is in a state of normal tonicity and shows absolutely no simultaneous activity with the relaxation of the urethra, bladder contraction, and fluid expulsion. The dog slept practically throughout the time that the record labeled figure 14 was being made.

With overdistention of the bladder the dog experienced forced respiratory movements. These, naturally, had their immediate effect on the vesicular pressure, just as they do normally. The optimum bladder rhythm was an independent series of contractions, which was not caused by any indirect influence from the respiratory or cardiac regions. The elongated respiratory waves upon which the cardiac rhythm is superimposed in normal pulse tracings are obtained as seen in figure 20.

#### THE TRANSFORMATION OF THE BLADDER MUSCULATURE

*Gross observations.* At the initial operation the bladder presented the appearance of smooth muscle on inspection. Very little resistance was met on cutting through the bladder wall, which measured 0.5 mm. in thickness. At the second operation the

TABLE 2

EXPERIMENT NUMBER	DATE MAY	LENGTH OF OBSERVATION	HEART PER MINUTE, AVERAGE	RESPIRATORY PER MINUTE, AVERAGE	BLADDER RATE PER MINUTE, AVERAGE
		<i>minutes</i>			
40	16	5	120	25	60
		10	125	30	80
		15	124	24	81
		5	128	31	85
		8	120	28	65
		6	130	33	110
41	17	2	125	29	75
		18	123	25	60
		20	124	35	125
		15	120	32	130
		5	121	30	135
		10	120	35	150
42	18	10	150	35	190
		10	148	28	75
		5	145	30	110
		20	155	29	90
		25	160	24	72
		5	158	26	80
43	19	5	135	20	40
		5	137	22	65
		5	136	28	60
		5	130	25	60
		5	139	24	70
		5	141	27	80
44	20	10	128	39	175
		5	134	35	150
		15	137	38	160
		3	135	40	160
		5	140	46	180
		10	144	45	170
45	21	5	169	27	70
		10	170	50	240
		18	175	65	304
		22	165	60	310
		5	160	55	290
		5	158	62	300

bladder musculature presented a deep red appearance, resembling somewhat cardiac muscle. The resistance to incision with the scalpel had greatly increased. The bladder wall was 5 mm. thick. The organ was highly vascular and the vesicular arteries and veins were abnormally enlarged when compared with those of a control puppy.

*Histological observations.* The excised portion of the bladder taken at the initial operation was normal smooth muscle of a developing bladder (figs. 9 and 10). The part of the bladder taken at the second operation for sectioning and study showed definite cross-striations and an increase in width and length of the muscle fibers over that of the control (figs. 11 and 12). The physiological reactions of a bladder that had been developed carefully to respond to increased work two hundred times greater than that which it was normally accustomed to, structurally reacted to do this work by the transformation of the smooth young muscle cells into striated muscle cells. The former type of muscle is incapable of doing the work accomplished by the bladder under observation, while the latter type possesses the capacity under the requisite stimulus for prolonged rhythmic contractions and increased work. The different degrees of energy possessed by the vesicular smooth and cross-striated muscles here studied is purely a biomechanical result corresponding to the differential amount of work that has been expended in their formation.

The complete study of the cytological and histological details revealed by the transformed striated muscle of the bladder will be reserved for a communication to be published later. The following may be noted: The bladder musculature prior to the experiment presented a syncytium with endoplasmic cells. The irregular, wavy, and twisted nuclei were found within the granular cytoplasm. The spongiosoplasm was drawn out in delicate longitudinal striations. The outer portion of the ectoplasm presents a modified surface layer; there is, however, no specialized sarcolemma. The endoplasmic units are comparatively narrow.

The cross-striated vesicular musculature presents broad fibers which form a network. The nuclei are centrally located and surrounded by granular endoplasm. The isotropic and anisotropic



cross lines are solid in some locations, in others granular. No intercallated discs, however, are seen.

The transitional epithelium of the bladder has undergone a hyperplasia (fig. 10). There are from ten to thirty layers of cells in the vesicular epithelium. The inner cells are greatly flattened and elongated in certain locations; no nuclei are seen in the layer bounding the lumen. In the greater part of the epithelium of the bladder the cells are nucleated from the basal to the inner group of cells.

Bardeen ('00-'07) and others have observed that the fibrillae found in developed skeletal muscle at first show no cross-striations. The deeply staining segment corresponds with the Q anisotropic band of the adult fiber and the other with the I isotropic striation (Warren Lewis, '12). The condition of the cross-striated bladder muscle corresponds to the granular alignment which forms subsequently the continuous Q bands illustrated during the development of cross-striated muscle by Godlewski ('02). The vesicular cross-striated fibers are formed from a syncytium composed of endoplasmic nucleated units. This is comparable to the developmental observations of McGill ('07) and Godlewski ('02) for smooth and striated muscles, respectively.

#### DISCUSSION

##### *Tension of differential growth as a stimulus to myogenesis*

According to the embryological evidence (Carey, '21), muscle formation in the gut is not due to a self-differentiation nor to a spontaneous self-elongation of the myoblast, but is a dependent modification of the mesenchyme, due to the tension elicited by an extrinsic growth force. In view of other evidence yet to be presented, the writer is confident that this is the fact as regards all musculature. For example, we may cite the spiral direction of the cardiac fasciculi corresponding to the changes in the vortical tension caused by the helicoidal blood stream flowing through the embryonic heart. In regard to the lingual musculature, an extrinsic force is found in the accelerated growth of the entodermal epithelium of the tongue.

The facts of direct observation prove that the formation of muscle tissue is a function of its position. Muscle tissue is formed *in situ* and is dependent upon an optimum tension, elicited by a dominant zone of accelerated growth, forcing by traction a connected zone retarded in growth. The direction of the resultant muscular fasciculi serves as a criterion of the direction of the dominant, accelerated force which exerted the tension of differential growth.

In view of the evidence supporting the conclusion that muscles arise through traction exerted upon the mesenchyme by a force extrinsic to the zone of myogenesis, it is as logical to claim that they self-elongate as it is to assert that a rubber band can stretch itself or that a balloon can self-dilate. In the latter cases extrinsic forces are implied. As regards muscle origin, these forces are elicited by extrinsic zones of accelerated growth, inevitably drawing by traction retarded zones of growth, this being due to their relative positions.

Facts previously presented by the writer prove that the developing descending colon of the pig embryo possesses two zones of differential growth which by their interaction mutually influence each other during the formation period. The inner epithelial tube is the dominant, most active region of growth. It presents numerous mitotic figures which pursue a path cephalad, primarily, in the manner of a left-handed helix. The outer mesenchymal zone is less active in growth and early in development is composed of a uniform mass of undifferentiated cells.

The inner, rapidly growing, epithelial tube practically revolves, due to the rapid spiral growth of its cells. With subsequent growth an apparent ring (this is qualified "apparent," for in reality a close spiral is formed) of smooth muscle myoblasts appears gradually near the periphery of the vortex.

This position taken by the inner, close spiral, smooth muscle coat at some distance from the epithelial tube is dependent upon an optimum tension. The attitude heretofore taken by the embryologists eliminated the search for the underlying cause of the first formed, inner muscle coat. The theory of self-differentiation excluded interpretation as regards myogenesis. A certain mesenchymal cell, regardless of position, was considered as destined to become a myoblast.

This highly differentiated tissue is now considered to self-develop, for so-called muscle-forming elements have been identified in the ovum. By the exclusion of this element in cutting experiments the subsequent positional environmental relation is as much destroyed as is the myoplasm. It may well be that the yellow pigmented zone is destined subsequently to assume a certain relation in development. This position may necessitate subjection to an optimum tensional-stress stimulus due to the differential growth. The conclusion of self-differentiation is consequently not entirely warranted and is too broad. All the work tending to support the generalization that muscle self-differentiates excludes the inner, environmental stimulus—the stretching or tensional stimulus of differential growth. Has the isolated myoplasm been cultured and found to form muscle? Only an affirmative answer to this question will warrant the assertion that muscles self-develop, and then only providing the exclusion of surrounding germ plasma has been accomplished so as to exclude totally tensional stresses of differential growth of relational parts.

The last statement is made since certain observers have concluded that muscle self-differentiates in experiments in which transplantation of tissues around the otic capsule of tadpoles were performed. That musculature subsequently appears is not to be wondered at, for the potencies or actualities of differential growth were also misplaced with the transplant. If the piece remained viable, it was certain to reveal subsequently the same tissue as in its normal location, for the resultants of differential growth and the potential mechanical stimulus due to space relation were left intact.

Consequently, the potencies of a blastomere are as much a function of its position as of its material substances. The material substances receive and react to the stimulus. The stimulus is a function of position. To elicit the response of mesenchymal cells in the formation of muscle tissue the proper optimum tensional stress stimulus must be applied. In tissue differentiation, therefore, the stimulus as well as the reception and response must be taken into consideration.

Tensional stresses are of various kinds and degrees. The quantity as well as the quality of stretching is important. The

connective tissues are resultants of certain degrees of stresses. Muscular tissues, on the other hand, are responses to still different types of stresses. The submucosa interposed between the epithelial tube and the inner smooth muscle coat presents cells which react to a certain minimum of tensional stress. Just peripheral to the submucosa, muscular tissue is differentiated as a response to an optimum tensional stress for muscle formation.

It was observed by von Uexkull that in the nerve net of invertebrates the excitation flows into a stretched muscle. Therefore, extension, stretching, or elongation of a muscle cell precedes the desired effective contraction, as was inferred long ago by Hunter from observations on mammalian muscular action. It was also found by Cannon that there was a subliminal, an optimum, and a supermaximal tensional stimulus to elicit the response of the contractile tissue of the stomach in its normal movements. Evidently, an analogy is here found for the development of the musculature. There appears to be a subliminal, an optimum, and a supermaximal tension for stimulating the formation of contractile tissue. In normal development as well as in subsequent normal function the tensional stresses appear to be fundamentally involved.

The writer has realized (J. Gen. Physiology, '20, vol. 3, p. 63) that the above direct embryological observations would be substantiated by experimentation, as is seen in the following quotation: "To an advocate of the experimental sciences it is undoubtedly necessary that an actual experiment should be made showing that by gradual stretching of a cell, under requisite circumstances, it is transformed into a muscle cell. To this end the writer is directing his attention. It must not be forgotten, however, that valuable suggestions pointing to a tensional stimulus as a factor in myogenesis are derived from a study of the origin of this tissue in a closely graded and advancing series of embryos. In the latter case direct observation reveals what is actually going on in nature's own laboratory."

The above consideration of tensional interaction of differential growth applies to the intestine. The same factor is revealed at work in the differentiation of the skeletal muscles. The primordial blastemal skeleton is undergoing the most rapid growth, as a conse-

quence of which a tensional elongating or stretching action is bound to be exerted upon the surrounding and less actively growing continuous syncytial mesenchyme. It is desired, therefore, to emphasize the following facts:

First, that there is a dominant energy manifested by rapid skeletal growth.

Second, that this energetic zone exerts a tensional or stretching action upon the surrounding mesenchyme, influencing the first steps of myogenesis.

Third, that the first differentiated muscles react upon the primordial blastemal skeleton resulting in a definite series of changes. These are seen in the formation of the condensed cartilaginous skeleton and later, as the muscles become more developed and vigorous, in physiological function in the formation of the osseous skeleton.

This action and reaction of forming parts results in the condition that at any period of development the degree of differentiation of the musculature and skeleton represents an equilibrium established between opposing myogenic and skeletal forces. Mechanically, therefore, skeletal and the related muscular tissues are interdependent, one relying upon the other for its initial and continued differentiation. The interaction of the growing parts in the embryo is considered in an exposition based on experimental teratological evidence by Stockard ('21).

#### *Reciprocal elongation of muscles*

The contracted state of a muscle as well as the relaxed arises from a power inherent in itself. The elongation or stretching superimposed on a muscle in tonic contraction depends on some extrinsic power. Simple relaxation of a contracted muscle is not sufficient to enable it to produce another requisite effect. It is necessary that there should be an elongator equal to the quantity of contraction intended to be produced. No muscle has the power of adequately extending or stretching itself; therefore, there must be an elongator. The elongators are usually muscular, but elastic tissue may serve this function as well as fluids in musculo-tubular organs, like the bladder.

The reciprocal elongation of muscles is strikingly evident in the intestine. Rhythmic contraction is due to a reciprocal mechanism; each wave is composed of a contraction and an elongation of the inner spiral coat alternating with a contraction and an elongation of the outer elongated spiral or longitudinal muscle coat. At the start the contraction waves of both coats begin together, but, due to the rotary course of the inner wave and the translatory course of the outer wave, the former and stronger one will inevitably trail the latter and weaker one. The outer and inner muscles are reciprocal elongators as peristalsis extends through the intestine.

When the outer and inner muscles are in normal tonic equilibrium, no distortion is evident. As soon as a contraction wave starts the balance is upset. The stronger cephalic constriction causes an elongation of the outer muscle coat. The wave of the latter follows in the path of the distal region of elongation. The contraction of the outer coat causes an elongation of the inner coat in the region of the caudal dilatation. Subsequently, the contraction wave of the inner coat is seen to occupy the former zone of stretching in the region of the caudal dilatation. There is, therefore, a definite syncopation in the activity of the outer and inner muscle coats as the peristaltic wave travels through the intestine. The muscle coats act as reciprocal elongators; consequently, peristalsis progresses for a variable distance through the gut instead of coming to a dead center.

In the heart the muscle layers are wound in complex spirals. Some of these spiral layers are elongated like the outer coat, others closely wound like the inner and intermediate layers (McCallum, '98; Mall, '96). It has been previously observed by Erlanger ('10-'12), Erlanger and Blackmann ('07), Garrey ('11), Moorhouse ('12), and others that various parts of the heart, especially the veno-auricular portion, when isolated in strips show automaticity. The ventricle possesses this property also when strips composed of at least two spiral layers with the long axis of the musculature running in different directions are isolated. If, however, a thin strip from the periphery of the heart is isolated it will not show rhythmicity if the outer layer of muscle composed of fibers running in the same direction has been stripped off. In the heart, there is

a reciprocating tensional interaction of the muscle layers. This reciprocal elongation is not as strikingly manifested in the heart as in the small intestine because the former organ is composed of muscle layers forming a more compact and complex spirality than the latter one (Carey, '21). That the rhythmicality is lacking in irritable strips composed of fibers only running in the same direction is convincing proof of the reciprocal elongation of the muscle layers. It is the muscular arrangement and their functional tensional interaction in the isolated heart that accounts for cardiac rhythmicality when the tensional stimulus of the blood stream is released and not some mysterious series of hypothetical explosion.

*The extrinsic tensional stimulus for the genesis of rhythmic beats*

The tensional interaction of the contained fluids, namely, the blood and the urine on the cardiac and vesicular mesenchyme, respectively, will be reserved for detailed presentation in a future communication. It will suffice to say at this point as regards the development of the chick heart that the two main cardiac muscle layers, an inner close spiral, and an outer open spiral layer are produced by the heliloidal blood stream flowing through the heart beginning at the sinus venosus at the confluence of the two omphalomesenteric veins. The inner cardiac layer is formed when the heart is growing relatively more rapidly in width than in length; the outer open spiral layer when the heart is growing more rapidly in length than in width. The blood stream is the extrinsic efficient agent producing tension in the cardiac mesenchyme comparable to the epithelial tube in the intestinal mesenchyme. The force of the blood stream is many times greater, however, than that of the energy of growth of the epithelial tube. Hence the different types of muscular products.

The effect of the tensional action of the blood stream is red cross-striated muscle; of the intestinal epithelial tube, pale smooth muscle. Once the two cardiac groups of muscles have been wound up for action by the blood stream they present a reciprocal action in relation to each other. It is this reciprocal muscular action that keeps the heart beating for seven or eight days (provided the

cardiac musculature has not lost its irritability) after the circulating blood, which is the efficient tensional stimulus that stimulates and maintains the normal cardiac beat, has been removed from its sphere of action.

In considering the origins of the heart beat it is well to keep in mind two fundamental points: first, the stimulus, and secondly, the reacting body. The structure of the reacting as well as that of the stimulating body, however, contributes to the quality of the effect. As regards the initial heart beat in the chick embryo, the reacting body is the cardiac mesenchyme. This responds, due to its irritability, to the stretching stimulus of the accumulating and subsequently circulating blood. We may have a modified cross-striated muscular bladder like the subject of this experiment, but if the stimulus is lacking it has nothing to respond to, there is no rhythm. After the stimulus has been applied to the cardiac mesenchyme for a sufficient period, in order that the two spiral muscle layers may be differentiated, it will continue beating, due to the reciprocating interaction of the musculature, until its energy is expended. This is the factor of safety in cardiac muscle in case of a diminished volume of blood due to hemorrhage or other cause. The heart, however, may be completely inhibited in its rhythm if a sufficient volume of blood is withdrawn in acute hemorrhage. The automaticity may be reestablished by subsequent transfusion (McGrath, '14, fig. 8.). That the heart continues beating for a certain time, provided the irritability of the musculature is maintained, after the circulation has been released completely from the sphere of cardiac action, speaks no more for complete automaticity of the heart muscle than that a cuckoo-clock is completely automatic because it will run for a definite period after the key that wound up its springs has been removed from the sphere of rhythmicity.

We may analyze the heart all we wish, but we are dealing with the irritable reacting body. The extrinsic stimulus that gives rise to the 'inner impulse' that causes the beat has not been touched in the problem. The neurogenic as well as the myogenic theories of the heart beat have left untouched the extrinsic stimulus—the circulating blood. It is the circulation that initiates and maintains the heart beat normally in the chick embryo as well as differentiates



the cardiac musculature by a dynamic tensional interaction. The cardiac musculature responds to the optimum stretching stimulus by contraction, thus the circulation is maintained—the blood is driven on. There is a definite mechanical interaction between the circulating blood and the responding heart in contraction.

Whatever chemicals are withdrawn from the circulation that are needed to maintain the normal cardiac irritability are bound to affect the beat. All the chemical work that has been accomplished on the heart beat does not vitiate or exclude the extrinsic mechanical, tensional stimulus applied by the circulating blood. As regards the extrinsic tensional stimulus of the heart beat, it is haemogenic in nature and, as we have seen definitely, for the striated muscular bladder rhythm it is hydrogenic in nature. We saw in the bladder that the beats were inhibited completely by withdrawing the stimulus. The bladder musculature is not wound up in a manner like the heart, in which the muscle layers are related synergistically for reciprocal interaction; therefore, it does not present automatic attributes.

The nervous mechanism regulates and helps to maintain cardiac irritability. The nodal tissues of the heart belong to the reacting body. Any structure intrinsic in the heart contributes merely to the second act in Newton's third law of motion, i.e., the reaction. The primary activator, initiator, or stimulus is the extrinsic, mechanical, hydrodynamic pressure tension produced by the circulating blood which causes tension of the heart muscle just as the water pressure is the stimulus for the rhythmic action in the hydrant force-pump.

#### CONCLUSIONS

1. *The differential degree of energy possessed by the types of muscle is purely an embryological biomechanical problem corresponding to the diverse amounts of optimum tensile work that has been expended in their formation by a dominant extrinsic energetic zone which draws out the premuscle mesenchyme in traction between the points of attachment at least one of which is mobile.*
2. The elongation of the muscular fasciculi is in the direction of a dominant force extrinsic to the zone of myogenesis, just as the strands of a mass of taffy candy are in the direction of the diverging supports—the hands.

3. *The essential difference between the pale smooth muscle of the bladder and the red involuntary striated muscle of the heart is dependent upon the differential intensity of hydrodynamic tensional stimuli to which the vesicular and cardiac mesenchymal syncytia, respectively, have been subjected during development.*

4. *The evidence herein presented proves definitely that the pale bladder musculature may be transformed into the red, cross-striated type by increasing the tensional stimulus to a degree comparable with that which the cardiac mesenchyme experiences normally.*

5. Muscle tissue is not a self-differentiated product, but is a bio-mechanical resultant of an optimum tension. The variable intensity of the optimum tension determines the muscular type. The growing cells receive and respond to the mechanical tensional stimulus. The stimulus, however, is a function of position.

6. In considering the origins of the heart beat, the extrinsic hydrodynamic tensional stimulus as well as the irritable reacting body—the heart muscle—is shown to be absolutely necessary as one of the factors accountable for heart rhythm.

7. The evidence herein presented proves that the structure of striated muscle is determined by the function it performs and the work it does and that cross-striated muscle is not formed in anticipation to a future function. The conclusion is warranted that function in this case determines structure, and not the reverse.

In addition to assistance received from his wife and Dr. McGrath, as mentioned above, the author is also indebted for help to Dr. Benj. Schlomovitz and Mr. Leo Massopust, the departmental artist. To Dr. C. R. Bardeen, of the University of Wisconsin, the author's thanks are due, for his most encouraging interest in this problem.

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## PLATES

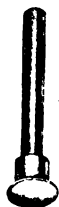
## PLATE 1

### EXPLANATION OF FIGURES

- 1 Silver suprapubic tube with flange which was sewed into the bladder.
- 2 Silver piston with cap which was screwed into the suprapubic tube when the experiment was not being performed.
- 3 Piston encased in flanged tube.
- 4 Cap of piston exposed, in situ, on the ventral abdominal wall of the four weeks-old puppy.
- 5 X-ray of the suprapubic tube and piston, in situ, ventral view.
- 6 X-ray of the suprapubic tube and piston, in situ, lateral view.



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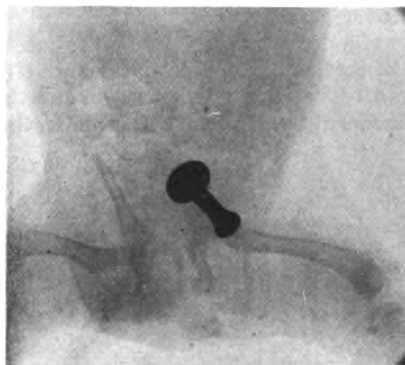
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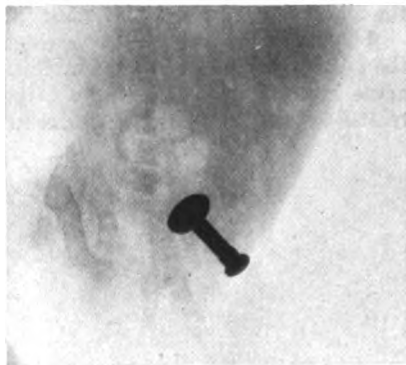
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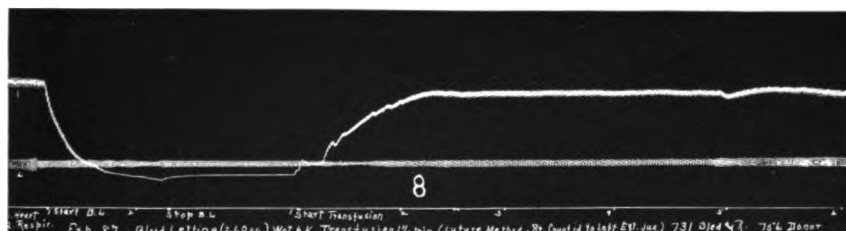
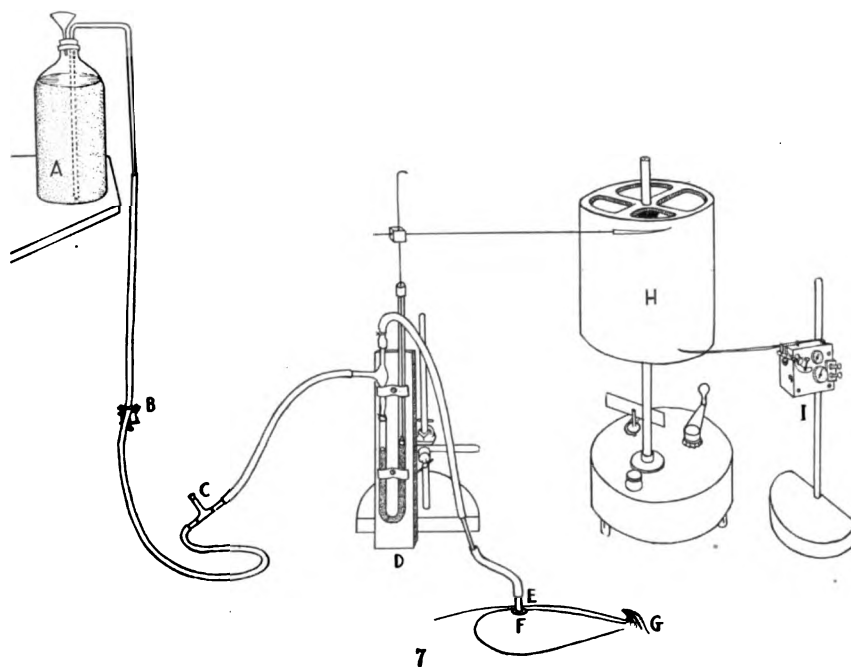
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## PLATE 2

### EXPLANATION OF FIGURES

7 Outline drawing of apparatus used in experiment. *A*, Elevated pressure reservoir filled with concentrated boric-acid solution at 37.5 C.; *B*, Screw-clamp; *C*, Mechanical valve; *D*, Mercury manometer; *E*, Inlet tube leading to bladder through the silver suprapubic tube; *F*, The flanged end of suprapubic tube within the bladder; *G*, Urethra; *H*, Drum; *I*, Timer.

8 Pulse (1) and respiratory tracings (2) of a dog weighing 6 kg. The drop in the pulse tracings to complete inhibition of cardiac beats is clearly seen, due to acute hemorrhage; 260 cc. were withdrawn. The rise in the pressure and the reestablishment of the rhythm is due to subsequent transfusion. (After McGrath.)





### PLATE 3

#### EXPLANATION OF FIGURES

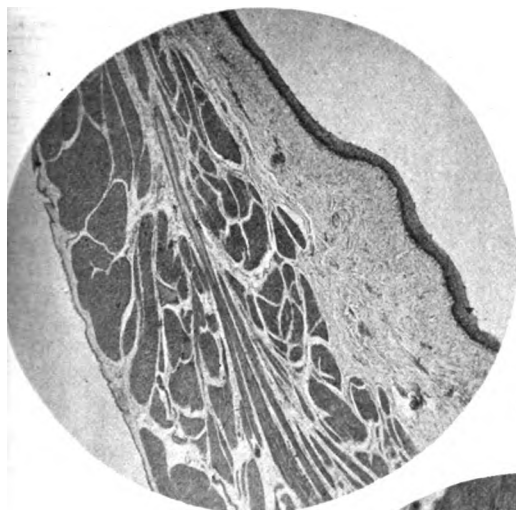
9 Microphotograph of bladder prior to experiment. The characteristic arrangement of the smooth muscle and the transitional epithelium are clearly seen.  $\times 42$

10 Microphotograph of same bladder forty-eight days after experimentation. The bladder was subjected to 200 times more work than it normally experiences. The smooth muscle was transformed into striated muscle in response to increased hydrodynamic tensional stimuli. The transitional epithelium hypertrophied into the stratified squamous type.  $\times 42$  B. & L. objective 16 mm. ocular 5.

11 Microphotograph of smooth muscle of the bladder prior to the experiment March 30, 1921. No cross-striated muscle is seen.  $\times 171$

12 Microphotograph of the transformed cross-striated bladder muscle May 21, 1921.  $\times 171$ . B. & L. Obj. 4 mm. 0.85 ocular 5.

13 Microphotograph of cardiac muscle.  $\times 84$ .



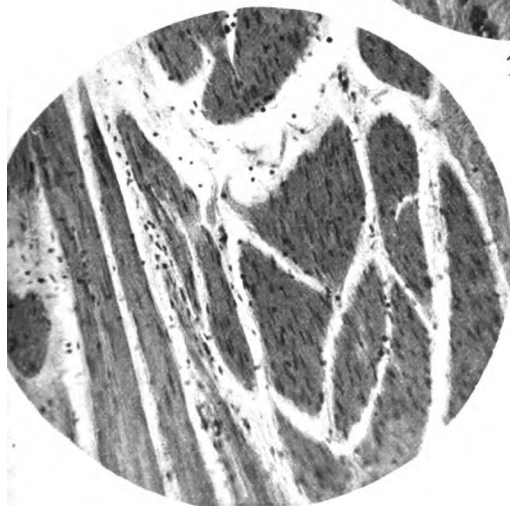
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## PLATE 4

### EXPLANATION OF FIGURES

14 Lines 1, 2, 3, 4, and 5 represent the variable response of the bladder to a constant hydrodynamic tensile stimulus for the first one hour and fifteen minutes of observation on May 21st, from 7 to 8:15 A.M., after the dog had had a night's rest of ten hours. At the beginning of the line the bladder made about six vigorous contractions a minute, whereas at the end of the line 1, bladder was contracting at the rate of 30 beats per minute. The average rate in lines 2, 3, 4, and 5 is 55 beats per minute. Mercury manometric pressure curves.

15 In lines 1, 2, 3, 4, the vesicular rate of contraction averages 60 per minute. In lines 5 and 6 the rate was accelerated by slight increase of pressure to 80 per minute. By a slight decrease in fluid pressure, line 7, the rate returned to 60 per minute.

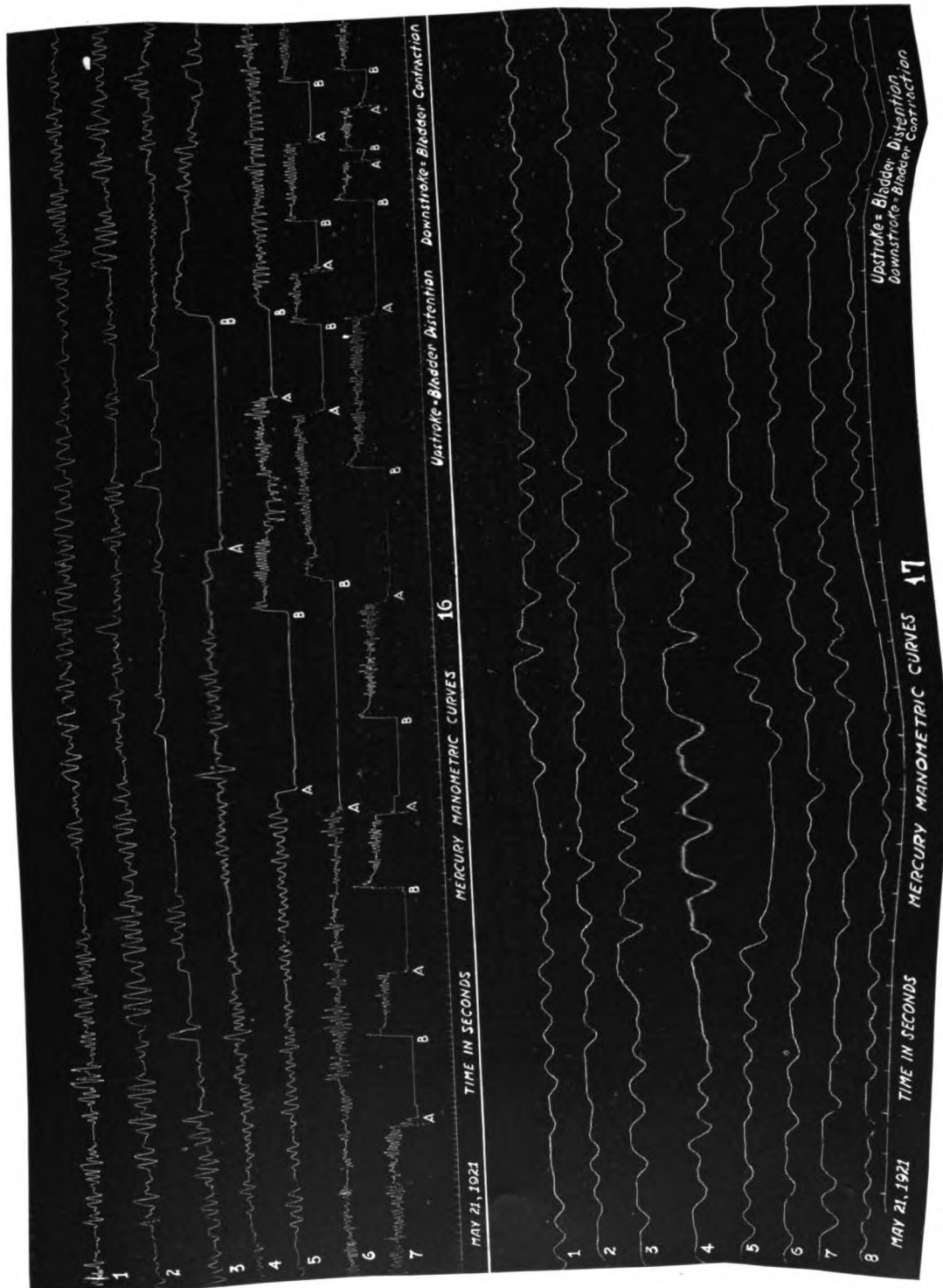


## PLATE 5

### EXPLANATION OF FIGURES

16 Lines 1, 2, 3, 4, 5, 6, and 7 represent the variable response of the bladder to a variable pressure stimulus. The rate of contraction in lines 1 and 2 average 50 per minute. From *a* to *b*, lines 4, 5, 6, and 7, the fluid circulation was completely inhibited. Concomitant with the lack of the tensile stimulus *a* to *b* there is a lack of vesicular beats, the pressure drops as noted by the lowered straight line. From *b* to *a* the pressure was suddenly raised. Note the immediate rise in pressure and the acceleration of vesicular beats. The average rate of contraction in line 7 and after the circulation is suddenly established in the bladder is 300 per minute.

17 This record shows the structure of the pressure curves on a fast drum. The up-stroke represents increased pressure simultaneous with the distention of the bladder; down-stroke, decreased pressure concomitant with contraction of bladder and expulsion of fluid through the urethra. These curves are of different types; some are single, others double hillocks, while some show the pressure rising gradually and then a sudden release with the contraction of the bladder. Certain curves show a comparatively acute summit, others a definite plateau. This indicates a variable irritability and response of the modified vesicular musculature to the constant extrinsic tensile stimulus.

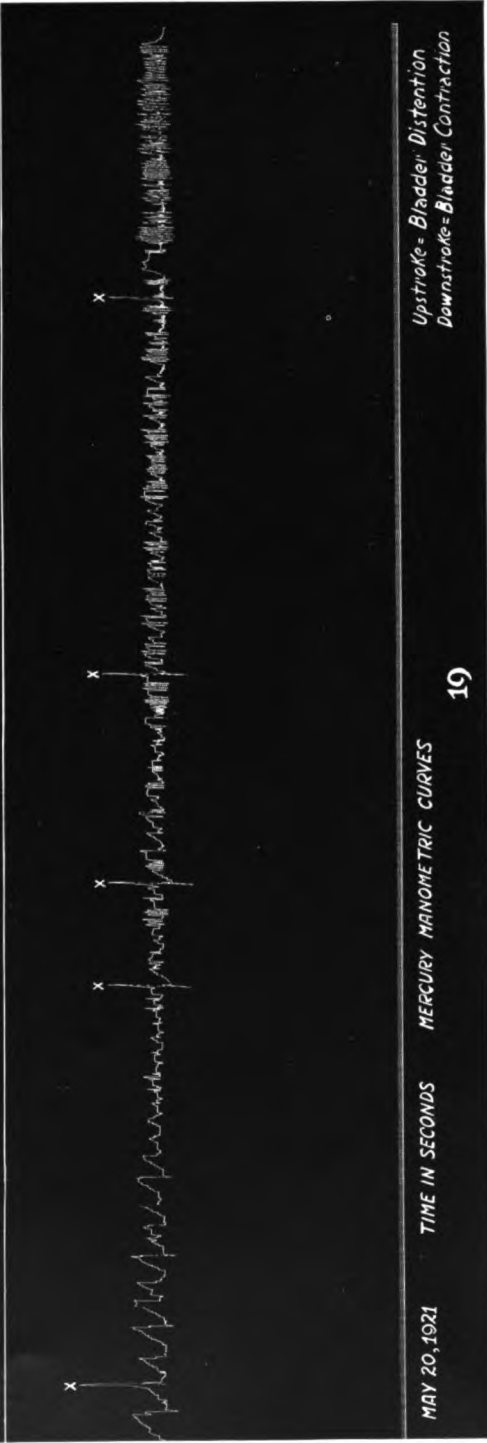
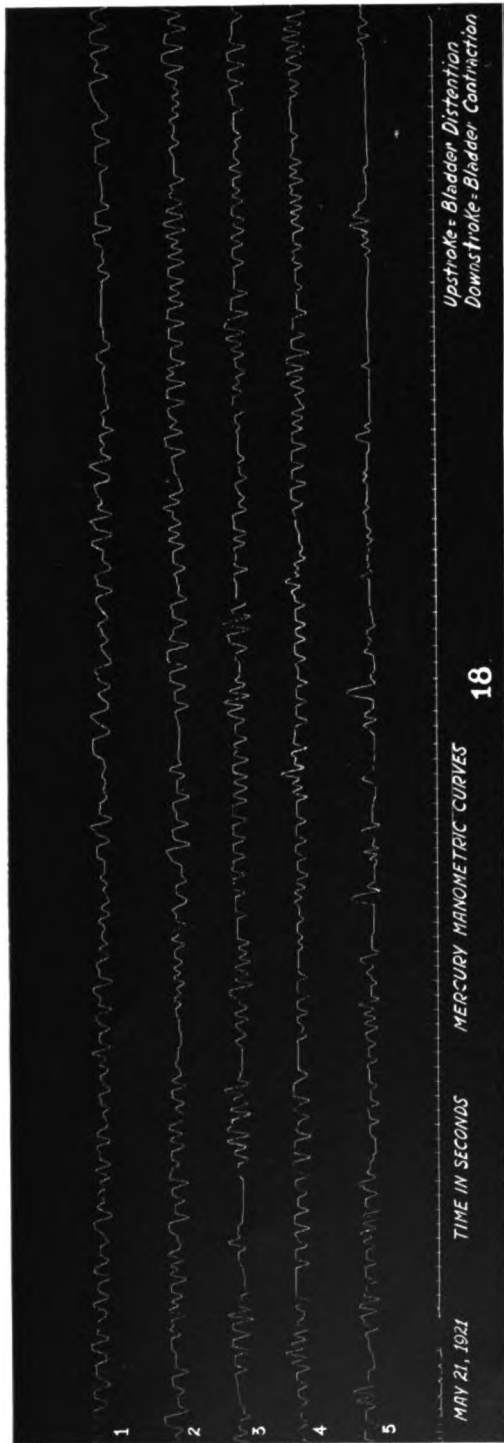


## PLATE 6

### EXPLANATION OF FIGURES

18 This record is a continuation of the graphic observations made in figure 16 on a faster drum. The structure of the various curves is clearly seen.

19 This record was made with no mechanical valve between the elevated pressure reservoir and the mercury manometer. Note the staircase appearance of the increasing pressure curves. This is produced by a constant flow of fluid into the bladder. Each attempt at bladder contraction is followed by distention due to the constant increase of fluid pressure within and resulting tension of the bladder. This continues in a summated manner until the force of bladder contraction completely overcomes the hydrodynamic pressure.





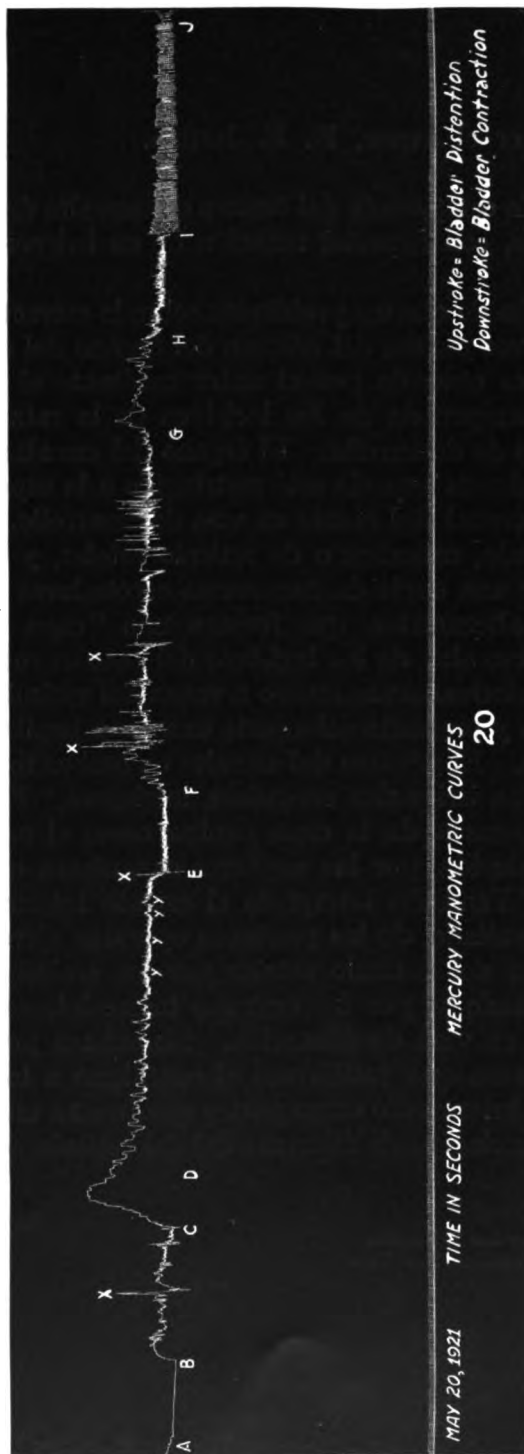
## PLATE 7

### EXPLANATION OF FIGURE

20 This record shows the straight line *a* to *b*, during which no fluid was flowing into the bladder; the pressure is represented by a straight line. From *b* to *c* the pressure was slightly increased and from *c* to *d* still further increased. From *d*, *e*, *f*, the pressure was diminished and the full more regular bladder responses are clearly shown *g* to *j*. From *c* to *d* the rate of bladder contractions was 120 per minute, from *i* to *j*, 55 per minute. The augmented contractions at *x* represented a superimposed forced inspiratory action. At *y* the respirations are more regular and cause the bladder contractions to be arranged in series of waves like the pulse.

DYNAMICS OF HISTOGENESIS  
EBEN J. CAREY

PLATE 7



Resumen por el autor, H. E. Jordan.

La histología comparada del órgano del esmalte del diente de los mamíferos, con especial mención de su irrigación sanguínea.

La solución de ciertos problemas sobre la estructura y función de las diversas partes del germen del órgano del esmalte de los mamíferos es atacada por el autor mediante el estudio de la histología comparada de los incisivos de la rata albina y del gato en vías de desarrollo. El órgano del esmalte de los incisivos de la primera especie está restringido a la superficie externa y el ápice coronal del diente en vías de desarrollo. Es relativamente delgado, careciendo de retículo estelar, y se caracteriza además por una serie de numerosas escotaduras y elevaciones irregulares alternantes, que forman una "capa papilar." El órgano del esmalte de los incisivos del gato envuelve a todo el diente, es relativamente grueso a causa del retículo estelar bien desarrollado y presenta solamente una capa papilar ligeramente desarrollada. El germen del esmalte en ambos tipos de dientes carece de una irrigación sanguínea intrínseca. El autor interpreta la presencia de la capa papilar como el resultado de la presión producida por los capilares sanguíneos que se aplican sobre ella. También interpreta al estrato intermedio como el principal resultado de las presiones opuestas ejercidas sobre la capa interna del retículo estelar por los ameloblastos que se están alargando y por los espacios intercelulares de la pulpa del esmalte en vías de dilatación. No existen pruebas histológicas adecuadas que justifiquen el considerar a la capa papilar o al estrato intermedio como partes que juegan un papel específico en la amelogénesis diferente del del retículo estelar en conjunto.

Translation by José F. Nonidez  
Cornell Medical College, New York

# THE COMPARATIVE HISTOLOGY OF THE ENAMEL ORGAN OF THE MAMMALIAN TOOTH, WITH SPECIAL REFERENCE TO ITS BLOOD SUPPLY

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Virginia*

SIX FIGURES (THREE PLATES)

## INTRODUCTION

This investigation seeks as its primary object to determine whether the enamel organ of the teeth of mammals is characterized at any stage of its development by an intrinsic blood supply. It aims further to explain the current confusion regarding this question. Among recent investigators, Williams, Hopewell-Smith, and Noyes claim that the enamel organ in certain higher mammals contains blood capillaries. Skillen, Jordan, and others claim that no blood vessels actually penetrate the enamel pulp ('stellate reticulum'). Skillen is unable to detect intrapulpal capillaries even in sections of injected specimens. Possibly the claim of an intrapulpal blood supply is based in part upon the observation of isolated blood corpuscles within the stellate reticulum. The presence of intrapulpal erythroplastids must be admitted. A complete solution of our problem demands, therefore, an explanation of the occurrence of occasional extravascular red blood-corpuscles within the enamel organ.

Not only as regards the question of presence or absence of blood vessels in the enamel pulp is there sharp difference of opinion, but disagreement also prevails concerning the depth to which the alleged intra-amelopulpal capillaries invade the stellate reticulum. Hopewell-Smith and Tims state that in the wallaby the capillaries which invade the enamel organ penetrate

only as far as the middle of the stellate reticulum. Williams concludes that in the tooth of the sheep the blood vessels penetrate as far as the stratum intermedium—a membrane surmounting the layer of ameloblasts. Hopewell-Smith makes a similar claim for the tooth of the kitten—a claim controverted by Jordan.

The question of the significance of the stratum intermedium also calls for incidental consideration in this connection. Williams is of the opinion that the cells of this layer select from the blood the materials out of which the enamel is elaborated. The facts, however, that a stratum intermedium is not present in typical form over the entire crown in certain mammalian teeth, that where it occurs typically it may extend below the level of enamel formation, that enamel formation begins before the complete development of this layer (Williams), and that it apparently does not exist in the enamel organs of reptilian teeth (Hopewell-Smith) cast considerable doubt upon this hypothesis.

The material of this investigation throws light also on the question concerning the function of that part of the enamel germ which extends below the level of the future neck of the tooth, that is, below the level of amelogenesis. Skillen accepts, and seeks further to support, the hypothesis of Röse that the presence of the ameloblasts is essential as a stimulative factor for the production of dentin. These investigators conceive of the non-amelogenic portion of the enamel organ as a 'stimulative layer' for the formation of the dentin of the subcervical portion of the tooth. That this hypothesis is inadequate, if not actually erroneous, is proved by the fact that in the case of the incisor teeth of the white rat the enamel organ never completely envelops the growing tooth, dentin forming nevertheless in the absence of any portion of the enamel organ over the inner surface of these teeth.

#### MATERIAL AND METHODS

The material chiefly employed in this investigation consists of sections of the head, in the region of the incisor teeth, of the white rat of from one to seven days after birth, and of sections of the lower jaw of the kitten, new-born and one, two, and three

weeks of age. The material was fixed in the Zenker-formol mixture of Helly, decalcified in a 2 per cent aqueous solution of nitric acid, imbedded in celloidin, sectioned at  $12\mu$ , and stained with hematoxylin and eosin. For assistance in the preparation of the sections I am indebted to two of my students, Mr. T. D. Jones and Mr. J. W. Hicks.

The reason for selecting this particular material is the fact that it is representative of two sharply distinct types of teeth classified on the basis of development (extent) of the enamel organ. In certain rodents with persistently growing incisors, of which the white rat serves well as a type, the enamel organ is limited in its extent to the outer surface of these teeth. The enamel organ of these teeth occurs, moreover, as a relatively thin layer, the stellate reticulum being practically lacking. In addition to these contrasting characteristics, it exhibits most conspicuously and in most highly developed form the so-called 'papillary layer' of Williams. The teeth of certain families of certain other mammalian groups (primates, ungulates, carnivores, and marsupials), of which the cat may serve as a type, are characterized in their development by extensive enamel organs completely covering the growing teeth. These enamel organs either lack a papillary layer or have it only very slightly developed. They are, moreover, of relatively great thickness, consisting of an inner enamel epithelium or layer of ameloblasts, a thin superjacent stratum intermedium, an extensive stellate reticulum, and an outer tunic of enamel epithelium. The most favorable approach to the problems relating to the enamel organ would seem to be by way of a comparative study of these two sharply contrasting types. Such procedure, providing an enlarged basis of definite and distinctive histologic data, gives greater promise of effecting an explanation and reconciliation of the conflicting opinions concerning the structure and function of the enamel organ.

## DESCRIPTION

*a. The enamel organ of the kitten*

The teeth of kittens of the first three weeks are completely covered by the enamel organ. Developmental conditions with regard to the incisors, canines, and the premolar teeth are very similar, and the following description applies in general equally well to either of these teeth. There are minor differences, especially with regard to the thickness of the enamel organ; that of the incisor teeth is somewhat thinner than that of the canines and premolars. Amelogenesis begins over the crest of the crown and progresses towards the neck. The enamel organ is accordingly more advanced in histogenesis over the coronal apex. It follows, then, that the enamel organ over the coronal parietes at any early stage of development represents an earlier histogenetic stage as compared with the coronal apex and a later stage as compared with the cervical and radicular portions. The prenatal condition of the enamel organ over the coronal apex may therefore be inferred from the postnatal condition of the organ over the subcoronal portions of the tooth. It should be emphasized that in the kitten's tooth the enamel organ envelops practically the entire tooth, approximately to the level of the primordium of the future foramen apicis dentis. Judging from the published illustrations, a similar condition prevails also at comparable stages in the teeth of man, sheep, dog, pig, calf, and wallaby. A description of the enamel organ of the kitten will therefore apply generally also to other large groups of mammals.

The enamel organ at this early postnatal stage is thickest in a wide region between the coronal and radicular apices, that is, in the region of the primitive neck (fig. 1). Here it consists of an innermost layer of tall columnar cells, the ameloblasts, a wide pulpar region or stellate reticulum, and a thin superficial layer composed of cuboidal or squamous cells of an endotheloid character (fig. 2). Between the layer of ameloblasts and the stellate reticulum there occurs, more or less regularly, a thin layer of closely packed small ovoid cells, the stratum intermedium.

These cells are in intimate structural relation with both the ameloblasts and the cells of the stellate reticulum. The stellate reticulum is composed of widely separated stellate cells, the intercellular spaces being filled with a basophilic tissue fluid. This so-called enamel pulp resembles somewhat the gelatinous connective tissue of the young umbilical cord. In contrast with the basophilic staining reaction of the stellate reticulum, the stratum intermedium has an acidophilic reaction, staining a deep pink or light red color in the eosin counterstain. The outer enamel epithelium is likewise acidophilic in staining reaction. In the tooth of the cat the stratum intermedium is present as a layer of compact cuboidal or ovoid cells along the entire extent of the enamel organ except in the extreme radicular portions.

Great importance has been ascribed by Williams and others to the so-called papillary layer. It becomes necessary to describe this layer in some detail. Though much better developed, and therefore more conspicuous, in the rat's incisor tooth (fig. 6), it is clearly present also in the teeth of the kitten (figs. 1 and 2). At the stage here considered it occurs only over the surface of the crown. It consists simply of a collection of irregular projections and ridges (resembling papillae in sections) on the outer surface of the enamel organ of the dental crown. It involves both the outer tunic of enamel epithelium and a variable depth of the peripheral portion of the stellate reticulum. It is therefore not a distinct layer, but simply a modified peripheral portion of the enamel organ. This modification, consisting of an alternation of irregular grooves and ridges, results from the operation of the mechanical factor of unequal pressure, causing indentations, in certain regions. This pressure factor inheres in the abundant blood capillaries which abut upon the surface of the enamel organ. In short, blood vessels produce numerous indentations in the coronal surface of the enamel organ, effecting thus a modification in the peripheral portion, to which the name 'papillary layer' has been applied by Williams.

The intimate relation of these circumdental capillary blood vessels to the enamel organ must be further considered. These



capillaries end abruptly upon the surface of the enamel organ; they nowhere penetrate the stellate reticulum. Occasionally the condition may be seen in sections where a capillary has indented the outer surface of the enamel organ and is then continued along the surface vertically for a long distance, following various irregularities of the outer tunic (fig. 2). The picture suggests a process of deflection of a blood vessel met with the resistance of an opposing obstacle. Occasionally one sees what appears to be a blood vessel within the outer border of the enamel pulp. When such a vessel is carefully traced, however, it becomes clear that the vessel has not actually penetrated the enamel organ, but has simply pushed itself into the periphery, carrying the outer tunic before it. Blood vessels do not invade, but simply invaginate, the peripheral surface of the enamel organ.

The extensive network of extrinsic capillaries in their ramification over the surface of the enamel organ, and pressing upon the outer tunic, becomes so intimately blended at certain points with the outer enamel epithelium that the endothelium and the enamel cells become fused into a continuous membrane. Indeed, probably also chiefly through the operation of the factor of mutual pressure, the outer enamel epithelium and the mesenchymal cells of the dental sac also become continuous. Where the endothelium of the terminal capillaries and the cells of the outer enamel epithelium fuse, opportunity is offered for blood corpuscles to pass into the outer border of the enamel organ, mainly into the meshes between the outermost border of the stellate reticulum and the outer enamel epithelium. Such corpuscles then come to lie free in the intercellular spaces. But neither the capillary wall itself nor the capillary lumen as such is continued into the enamel organ. The presence of such isolated red blood corpuscles in the peripheral portions of the enamel germ has no doubt given rise in part to the erroneous interpretation of intra-amelopulpar capillaries. The observation of such intercellular corpuscles and of invaginated blood vessels (giving the appearance of intrapulpar vessels in certain sections) has led to the mistaken impression that blood vessels penetrate the enamel organ. The intercellular blood corpuscles of the enamel

organ, resulting from the establishment of continuity between the capillary lumen and the intercellular spaces of the outer enamel epithelium, following fusion of the endothelium with the latter cells, are in effect foreign bodies in the enamel germ. They are, moreover, treated by the enamel germ as foreign bodies. Groups of cells of the outer enamel epithelium fuse to form giant-cells which ingest these extravascular erythroplastids.

Within the enamel pulp occur also spherical and oval globules of varying sizes, especially abundant near the border of the stratum intermedium. These globules were described in a previous paper, and there tentatively interpreted as superfluous enamel secretions on the part of the potential ameloblasts of the stellate reticulum. These globules also are ingested and resorbed by giant-cells, formed in this case by a fusion of cells of the stratum intermedium.

As regards the stratum intermedium, its formation seems the result also chiefly of the operation of the mechanical factor of pressure. Apparently the mutually opposing pressures of the elongating ameloblasts and the expanding intercellular spaces of the stellate reticulum cause a compression and consequent flattening of one or several layers of cells (representing the less differentiated layers of the germinative portion of the original oral epithelium) between them, producing thus the so-called stratum intermedium. This interpretation makes intelligible the variable presence of the stratum intermedium in typical form throughout the enamel organ.

*b. The enamel organ of the white rat*

When we consider, now, the enamel organ of the rat's incisor, as compared with that of the kitten's tooth, we are struck at once with sharp contrasts. In the first place, the enamel organ of the rat's incisor covers only the outer surface of the tooth, including the coronal apex (figs. 4 and 6). A section through the primitive foramen apicis dentis shows a condition essentially like that of other mammalian teeth (fig. 3). In the second place, this enamel organ is relatively very thin. A typical stellate reticulum is lacking (fig. 5). The inner layer of ameloblasts,

a thin stratum intermedium, and an outer tunic occur essentially as described for the kitten's tooth.

A third differential characteristic concerns the extensive indentation or furrowing of the enamel organ of the rat's incisor. The impinging capillaries push the outer enamel epithelium into contact with the stratum intermedium, so that the resulting 'papillae' of the 'papillary layer' consist essentially of clusters of cells of the outer tunic and the stratum intermedium (fig. 6). The cells of the outer tunic have taken on a more or less regular fusiform shape, and the 'papillae' as a whole have an acidophilic staining reaction. The papillary layer of the enamel organ of the incisor of the rat accordingly involves the entire thickness of the organ. It results from an inpushing of capillaries. Just as in the case of the enamel organ of the cat, so in the rat the endothelium of the capillaries at certain points may fuse intimately with the cells of the outer tunic. This effects fenestration of the capillary wall and a continuity of capillary lumen and intercellular spaces, and as a result permits an infiltration of blood corpuscles. Since the enamel organ as a whole is here very thin, these extravasacular erythroplastids penetrate as far as the stratum intermedium. But no capillaries as such actually pierce the enamel organ. Here also these intercellular red blood corpuscles may be ingested by giant-cells formed from the cells of the enamel germ.

A clear understanding of the histologic relations of the enamel organ to the developing incisor of the white rat demands a consideration of the grosser features of these teeth. In the lower jaw these two teeth grow almost horizontally in line with the long axis of the jaw; they are only slightly curved. A transverse section of the head and jaws will therefore cut these teeth approximately transversely. The two incisors of the upper jaw, on the contrary, curve sharply along the lateral border of each nostril, tracing in their growth a complete semicircle from base of papilla to point of eruption. Thus at the root the direction of the tooth is vertical, alongside the nostril it is almost horizontal, and terminally it again turns almost into a vertical position. The concavity of these curving teeth is of course on the lingual

surface. Transverse sections of the head and the upper jaw at the level of the dental papilla will therefore show an approximately longitudinal (frontal) section of the radicular portion of this tooth, the enamel organ appearing to cap the coronal surface (fig. 3). The tooth as seen at this point appears to be growing towards the top of the head. Sections farther forward will yield approximately transverse sections (like that of fig. 4), while sections towards the tip of the jaw will again give approximately longitudinal sections of these teeth. The enamel organ is accordingly not restricted to the crown of the tooth, as sections through the radicular area would lead one to infer, but actually to the upper (outer) surface of the tooth. The enamel organ is in fact continued over the coronal apex, but posteriorly its ameloblasts early undergo regressive changes essentially like those described by Addison and Appleton ('21) for the molar teeth of this same species. Accordingly, enamel is not formed over the coronal apex nor along the inner surface of these incisor teeth. The absence of enamel organ along the inner surface of the upper incisors, and the resulting intimate relation between the forming dentin and the mesenchyme of the dental sac may be correlated with the later continuity along the ventral border basally of the teeth with the alveolar bone. Likewise, the curved manner in which the enamel germ invades the jaw and is met obliquely by the dental papilla, the latter invaginating the former in such manner as to remain bare of enamel organ along the inner surface, is correlated with the future greatly curved form of these teeth and their lack of enamel on the posterior (inner) surface. These peculiar incisor teeth of rodents could be homologized with the more usual form of mammalian teeth by interpreting the outer enamel-covered surface as a greatly elongated crown, the actual chisel-edged and enamel-free crown and the dentin-covered inner surface constituting the subcervical portion of the tooth.

## DISCUSSION

*a. General*

The fundamental cause of the structural differences in the enamel organ of the incisor teeth of the white rat and of rodents in general (with the exception of the suborder, *Lagomorpha*, including rabbits and hares) as compared with those of other mammals, inheres in the fact that the rodents' incisors grow persistently throughout life. They lack a genuine root, having a persistent pulp; and they have no deciduous predecessors. The restriction of the enamel organ of the rat's incisor to the outer surface of these teeth is correlated with a restriction of definitive enamel to this surface. The relatively thinner character of the rat's enamel organ, in which a stellate reticulum is practically lacking, may be correlated with the relatively more restricted and more compact dental sac, as compared with conditions in the cat, effecting in consequence greater pressure upon the enamel organ, to the suppression of development of a stellate reticulum. The absence of a stellate reticulum may perhaps be the result in part also of the greater attenuation of the enamel germ in these incisors, because spread over a larger surface, and more rapidly, in the case of these relatively long and rapidly growing teeth. The better developed character of the 'papillary layer' follows from the thinner condition of the enamel germ, and results from pressure on the part of impinging capillaries scattered over its surface.

The several differential characteristics of the enamel organ of the incisor of the white rat and the kitten throw much light upon the question of the significance of the enamel organ as a whole, and of its constituent layers: ameloblastic layer, stratum intermedium, stellate reticulum, and the outer enamel epithelium.

The function of the ameloblasts need not be discussed. It may be accepted as proved that they are the essential elements in the elaboration of enamel. As to how in detail the ameloblasts produce enamel prisms need not concern us here. We are interested chiefly in the question as to how the other layers of the enamel germ cooperate in this process.

*b. The papillary layer*

Williams was the first to direct special attention to the so-called papillary layer of the enamel organ. It is best developed in rodents. In the tooth of an embryo lamb Williams describes a marked tendency on the part of the cells of the stratum intermedium to assume 'a more or less orderly arrangement about the capillary loops.' He thinks it 'perfectly evident that in the development of enamel the cells of the stratum intermedium play the most important part in the selection from the blood of the materials for the construction of this tissue.' Williams accordingly derives the papillae of the papillary layer from the cells of the stratum intermedium. Skillen, on the contrary, derives them from an 'apposition of outer and inner tunics, which takes place gradually.' According to Skillen, calcification of the enamel prisms begins only at the time of the formation of the papillary layer. But Williams states that the formation of enamel begins before the full development of the stratum intermedium (therefore, before the papillary layer is formed), and that 'it is highly probable, as has been pointed out by previous writers, that the material for the commencement of enamel formation is stored in the stellate reticulum' (p. 118).

Williams describes the prisms of definitive enamel as the product of two distinct elements: 1) globules formed in the ameloblasts, probably directly from the nuclei; 2) 'an albumen-like appearing substance which is seen passing out of the ameloblastic cells of the stratum intermedium and evidently contains the mineral matter of which completed enamel consists' (p. 477). According to Williams, therefore, the stratum intermedium is of primary importance in amelogenesis. In it he claims are early developed numerous capillaries. In rodents the vascularized layer forms 'a highly differentiated secreting tissue.' The cells of the stratum intermedium become arranged in the form of papillae around and between which end abundant capillary loops. Each papilla is said to supply about twenty ameloblasts.

From the description of the comparative histology of the enamel organ of the incisors of the rat and of the cat it is clear that

the so-called papillary layer is not the same thing in both cases. It is, however, formed in exactly the same way and through the operation of exactly the same factors: pressure upon the outer surface of the enamel organ by impinging capillaries. The papillary layer of the enamel organ is accordingly simply a secondary mechanical modification of the peripheral portion of the enamel organ. In the cat's incisors, where the enamel organ is relatively thick, this modification involves only the outer enamel epithelium and the immediately adjacent peripheral portion of the stellate reticulum. It never involves the stratum intermedium, except, in an indirect way, in the later stages of development when the stellate reticulum has disappeared and the ameloblasts have practically ceased functioning. In the case of the rat's incisor, where the enamel organ is thin, due to the absence of a stellate reticulum, this same pressure of the capillaries upon the outer enamel epithelium pushes the latter into contact with the stratum intermedium, and in consequence the resulting 'papillary layer' involves all of the extra-ameloblastic layers of the enamel germ. Accordingly, contrary to the view of Williams and of Skillen, the papillary layer has no specific significance in amelogenesis distinct from the extra-ameloblastic portion of the enamel organ as a whole.

*c. The stratum intermedium*

This layer varies considerably in different teeth and in different regions of the same tooth. It is present over those areas where enamel is never formed as well as over the crown of the tooth. Being next the layer of ameloblasts, and in intricate continuity with these cells, as well as with the cells of the stellate reticulum, it of necessity is most closely concerned with the transportation to the ameloblasts of whatever material may be passed from the blood vessels through the stellate reticulum for the support of the metabolic processes of amelogenesis. Since in the incisor teeth of rodents the papillary layer is composed largely of cells comparable to those of a stratum intermedium, and in fact constitutes almost the whole of the extra-ameloblastic portion of the enamel germ of the crown, William's statement that the cells

of the stratum intermedium play the most important part in the selection from the blood of the materials for the constitution of enamel is correct in a general sense, but I am unable to detect any histologic evidence, such as presence of secretory granules, that this layer is actually a 'highly differentiated secreting tissue.'

The stratum intermedium is most probably to be interpreted as the result of mutually opposing pressures on the part of the growing tooth, with the lengthening ameloblasts, against the softer tissue of the enamel pulp. These opposing pressures of necessity involve primarily the cells of the original stellate reticulum next the outer ends of the ameloblasts. The modified character of the cells of the stratum intermedium, including cuboidal, ovoid, and stoutly fusiform types, and their closely packed condition, result from the operation of the mechanical factor of pressure, preventing a separation and the coincident differentiation characteristic of the cells of the stellate reticulum. The differential acidophilic staining reaction may be correlated with the homology of these cells with the cells of the rete mucosum of the epidermis. Their specific function in amelogenesis is, as far as can be judged from their cytology, not essentially different from that of the cells of the stellate reticulum as a whole. Amelogenesis cannot be dependent upon a specific stratum intermedium, for, as Williams admits, enamel formation begins before the layer is differentiated. Moreover, amelogenesis may be initiated and maintained in certain teeth in the absence of such a sharply defined layer.

#### *d. The stellate reticulum*

As regards the question of the function of the stellate reticulum in connection with amelogenesis, I can confirm Williams' observation of the formation of globules at the central ends of the ameloblasts; but I can discern no indication that these globules are derived from the nucleus. Of the derivation of an albuminoid mineral substance from the 'ameloblastic cells' of the stratum intermedium as described by Williams, I have seen no evidence in the sets of slides included in this study. However, in a previous study I ('21) described in the stellate reticulum



of the canine tooth of a newborn kitten certain spheroidal globules of various sizes scattered throughout the enamel pulp. These I tentatively interpreted as superfluous enamel globules formed by the cells of the stellate reticulum and the stratum intermedium. That they are of the nature of foreign bodies, however, seemed proved by the fact that some were ingested by giant-cells formed in the enamel pulp largely from the cells of the stratum intermedium. Possibly these globules are homologous with the albuminoid globules described by Williams in the stratum intermedium. Whatever value this substance may actually have in relation to enamel formation, its presence in the stellate reticulum suggests that this tissue has amelogenic capacity. However this may be, it seems clear on logical grounds that since the capillaries are excluded from direct contact with the ameloblasts, being separated therefrom by the varying width of the enamel organ at any point, the nutritive materials for the support of amelogenesis must be either transported from the extrapulpar blood vessels to the ameloblasts by the cells of the stellate reticulum, or a complete supply must have been originally stored in the stellate reticulum. The most plausible interpretation of available histologic evidence would seem to be that an original supply of amelogenic materials stored in the stellate reticulum is replenished peripherally from the extensive blood supply covering the outer tunic of the enamel organ. That this is probably the case is suggested also by the fact that in most mammalian teeth, at the stage of development at any point when the blood vessels come to lie nearest the ameloblasts, due to the disappearance of the stellate reticulum in the formation of the membrane of Nasmyth, enamel formation is complete. This interpretation is further supported by the fact that when a stellate reticulum is lacking, as on the rat's incisors, the blood vessels are brought relatively much closer to the ameloblasts.

*e. The relation of the ameloblasts to dentinogenesis*

This investigation is not concerned with the question of the origin of that membrane ('membrana preformativa') upon one side of which the ameloblasts deposit uncalcified enamel and upon the

other side of which the odontoblasts deposit dentin. Since the ameloblasts become differentiated and organized at any point before the odontoblasts, it might be assumed that the latter differentiate under the influence of a stimulus supplied by the ameloblasts. This is in fact the hypothesis formulated by Röse and supported by Skillen. Röse ('91) claims that as soon as growth of the enamel organ ceases rootward, dentin ceases to be formed. Skillen ('21) concludes that the enamel organ is a necessary antecedent of dentinogenesis, that it functions in addition to enamel elaboration as a 'stimulative layer' to odontoblast organization and dentin secretion. This hypothesis is sharply contradicted by conditions in the incisor teeth of the white rat. Here the enamel organ originally caps the dental papilla as in other mammals, but very early, as the tooth elongates, the enamel organ, while still enveloping the base of the persistent papilla, shifts to the front surface of the tooth and remains restricted to this side and the coronal apex of the preerupted tooth. This accounts for the presence of enamel only on the anterior (outer) surface of the rat's incisor. Nevertheless, dentin is deposited on the inner surface of these teeth in the absence of any representative of the enamel organ. Here again the factor of pressure, that is, adequate resistance or rigidity of tissue, seems to be the essential matter. The mesoblastic tissue of the dental sac is here so dense relatively that it offers the requisite resistance for the deposition and retention of the forming dentin. The supposition of a stimulative factor for dentinogenesis on the part of the radicular portion of the enamel organ seems to have no support from actual histogenetic facts.

*f. The blood supply of the enamel organ*

The central histologic datum of this investigation concerns the question of an intrinsic blood supply of the enamel organ. Where the enamel organ is relatively thin, as on the incisor of the white rat, the matter seems unimportant; for here the capillary net, though actually outside of the enamel organ, is nevertheless brought so close to the layer of ameloblasts as to be practi-

cally in contact with these cells. But in other mammalian teeth, where the stellate reticulum of the enamel germ is very extensive, the question of the blood supply for the ameloblasts takes on greater importance. For here the assumed nutritive materials for the support of amelogenesis, in the absence of intra-amelopulpal capillaries, must be transported by the agency of the cells of the stellate reticulum across a considerable width.

Skillen was unable to find, even in injected specimens, any trace of blood vessels in the enamel pulp or surrounding the stratum intermedium. He adds that 'when the outer and inner tunics come into apposition, thus forming the papillary layer, the vessels accompany the outer tunic and so come to lie in the portion described by Williams' (p. 3), that is, in the 'papillary layer' and in close relation with the ameloblasts. It must be remarked, however, that during the early stages of tooth development, when amelogenesis is at its height, the outer and inner tunics are still separated by a considerable area of stellate reticulum.

According to Noyes ('21), at about the time of the completion of the dental sac ('follicle'), there appear on the outer surface of the outer enamel epithelium ('tunic') small rounded projections of epithelial cells, and this layer becomes broken up coincident with an invasion of capillaries from the dental sac 'which carry the remains of the outer tunic down against the inner tunic to form the stratum intermedium. There is an intimate relation between capillary blood vessels and the stratum intermedium' (p. 332). Noyes accordingly regards the stratum intermedium as composed of pushed-in remains of the outer enamel epithelium, and concludes that capillaries extend directly into and through the enamel pulp ('stellate reticulum') to the depth of the stratum intermedium.

Aside from the fact that the stratum intermedium cannot be correctly described as composed of pushed-in remains of the outer enamel epithelium, I can find no evidence in my sections of an actual invasion by capillaries of the stratum intermedium or even of the stellate reticulum. Nor does it seem to me quite accurate to say that the outer enamel epithelium becomes broken

up coincident with an invasion of capillaries from the dental sac. As I have attempted to make clear in my description of conditions relating to the blood supply of the enamel organ, what actually occurs in the specimens of my sections is an intimate fusion at certain points between the cells of the outer enamel epithelium and the endothelium of the capillaries. This establishes a continuity between the lumen of the capillaries and the intercellular spaces of the outer enamel epithelium, permitting an infiltration of occasional red blood corpuscles. But no capillaries can be found actually growing into the enamel pulp. Areas giving the impression of intrinsic blood vessels are invariably found, on closer study, to represent transverse sections of capillary loops invaginated into the surface of the enamel organ. Such loops simply produce indentations of varying depth into the outer surface; they are always separated from the stellate reticulum, except for occasional restricted areas of fusion with the cells of the outer tunic, by the intact outer enamel epithelium. In other words, certain blood vessels may invaginate, but do not actually pierce, the outer surface of the enamel organ.

Hopewell-Smith ('18), while admitting that the external enamel epithelium and the enamel pulp of the tooth of the kitten lack blood vessels, claims nevertheless that one or two large non-branching vessels traverse the pulp and advance as far as the stratum intermedium where they 'suddenly break up into numbers of small capillaries, and form a beautiful plexus which supplies the cells of this intermediate layer and the internal epithelium' (pp. 263, 264). The illustrations offered in support of this description do not seem to me to warrant the interpretation given. Analysis of these illustrations (figs. 227 and 229) reveal that the capillaries end abruptly upon the outer enamel epithelium in that portion of the enamel organ where the stellate reticulum persists. Only over the coronal apex where the enamel organ remains only as a very thin membrane, consisting merely of largely spent ameloblasts and outer enamel epithelium, are the blood vessels brought in close relation with what represents the remains of the stratum intermedium. Here the enamel organ is atrophic, the tips of certain of the capillaries have fused

with the outer enamel epithelium, and occasional blood corpuscles have infiltrated the intercellular spaces of the area representing the former stratum intermedium. But no capillary endothelium has actually grown into this atrophic membrane.

In the case of the tooth of the wallaby, Hopewell-Smith and Tims ('95) make the still more positive statement that 'the abundant evidence of blood vessels within the enamel organ is a very striking feature.' They claim that blood vessels 'can be seen entering the enamel organ apparently at more than one point on the surface, and are often of sufficient size to clearly recognize the blood corpuscles within them. They can be traced to a point about midway between the outer and inner enamel epithelium, but we have not seen them proceeding farther, neither have we found them in the stratum intermedium, the position in which they were described by the authors above mentioned' (Poulton and G. B. Howes, in enamel organ of rat). They think that 'it is extremely probable that the unusual vascularity of the enamel organs in this animal (wallaby) is correlated with the precocious development of the enamel'.

But when one refers to their illustration of the tooth germs of the wallaby (fig. 216, Hopewell-Smith), one sees little in support of this statement. To be sure, some cellular mass, entering a very short distance below the surface of the outer enamel epithelium, and continuous with it, is labeled 'blood vessel.' But there is nothing shown to prove that this single vessel has actually pierced the outer enamel epithelium rather than having simply pushed it ahead of itself a short distance into the stellate reticulum. Even in the case of the enamel organ of the wallaby, where it is claimed the clearest evidence of intrapulpal capillaries is given, one can only remain skeptical about the actual presence of intrinsic blood vessels.

When one contrasts conditions in the dental pulp with those in the stellate reticulum of the enamel pulp one is further impressed with the meager evidence for the occurrence of blood vessels in the latter. The mesoblastic dental pulp, in contrast with the epiblastic enamel pulp, is extensively vascularized. There is not the slightest difficulty in tracing, in sections, cap-

illaries branching and ramifying even among the distal ends of the odontoblasts. It is the simplest matter to recognize and trace the finest capillaries of the dental pulp for long distances as they course among the odontoblasts even at right angles to the long axis of the cells. This being so, there should be at least not much greater difficulty in recognizing similar capillaries, if present, in the enamel pulp of the same sections. All the evidence, critically considered, supports the conclusion that blood vessels do not occur within the enamel organ of mammalian teeth.

#### SUMMARY

1. The comparative histology of the enamel organs of the incisor teeth of the white rat and of the kitten reveals decided differences. The enamel organ of the rat's incisor is restricted to the outer surface; it is relatively very thin, and becomes modified by the impingement of capillary loops into a papillated membrane. A stratum intermedium is not sharply differentiated. In the case of the kitten, the enamel organ covers the entire tooth, it is relatively thick, due to the presence of an extensive stellate reticulum, it contains a well-differentiated stratum intermedium, and papillae are limited to the extreme peripheral portion.

2. The restriction of the enamel organ to the outer surface of the developing incisor tooth of the rat proves that this tissue is not essential as a 'stimulative layer' for the production of dentin.

3. Neither in the case of the rat's incisor nor in that of the kitten's teeth is the enamel organ vascularized. Capillaries do not penetrate the stellate reticulum. They may indent the surface of the enamel organ, thus producing a 'papillary layer.' The tips of the capillaries may, especially in later stages, fuse at a certain point with the cells of the outer enamel epithelium, and so establish continuity between the capillary lumen and the intercellular spaces of the outer tunic. Red blood corpuscles may thus infiltrate the outer tunic. They are later ingested by giant-cells formed by the fusion of cells of the outer enamel epithelium.

4. Both the so-called 'papillary layer' and the 'stratum intermedium' are interpreted as essentially the product of the operation of the mechanical factor of pressure; the former resulting from the impingement of growing capillaries, the latter chiefly from the elongation of the ameloblasts.

5. There is no histologic evidence that either the papillary layer or the stratum intermedium plays a specific rôle in amelogenesis, distinct from that of the extra-ameloblastic portions of the enamel organ as a whole.

6. Apparent amelopulpar invasions of blood vessels are interpreted in terms of sections of such vessels pushing obliquely into the periphery of the enamel organ. Such vessels are still separated from the stellate reticulum by a double membrane composed of outer enamel epithelium and endothelium.

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## PLATES

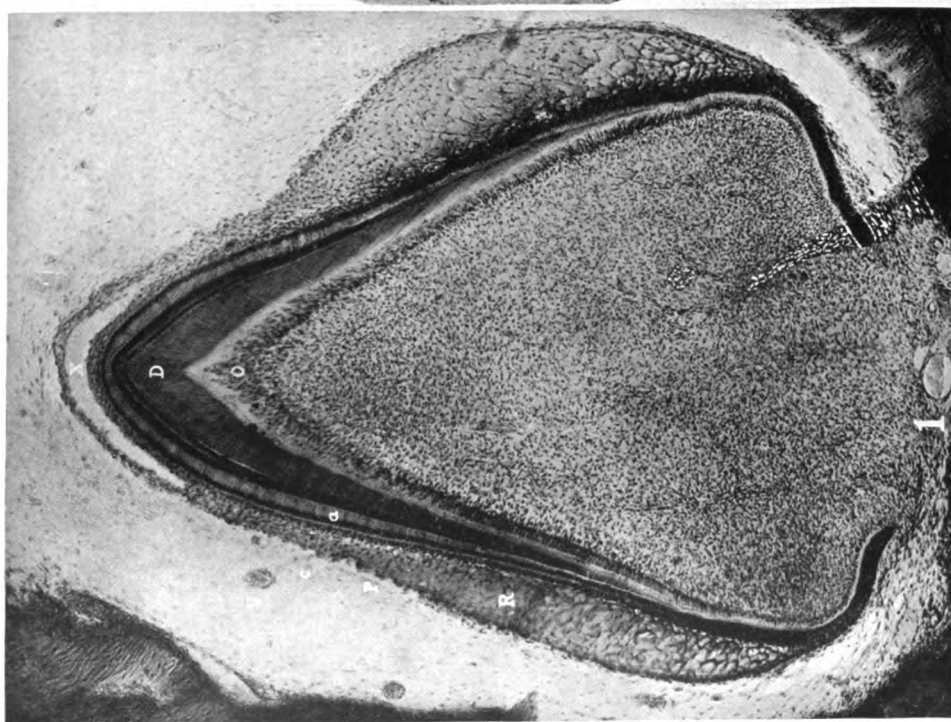
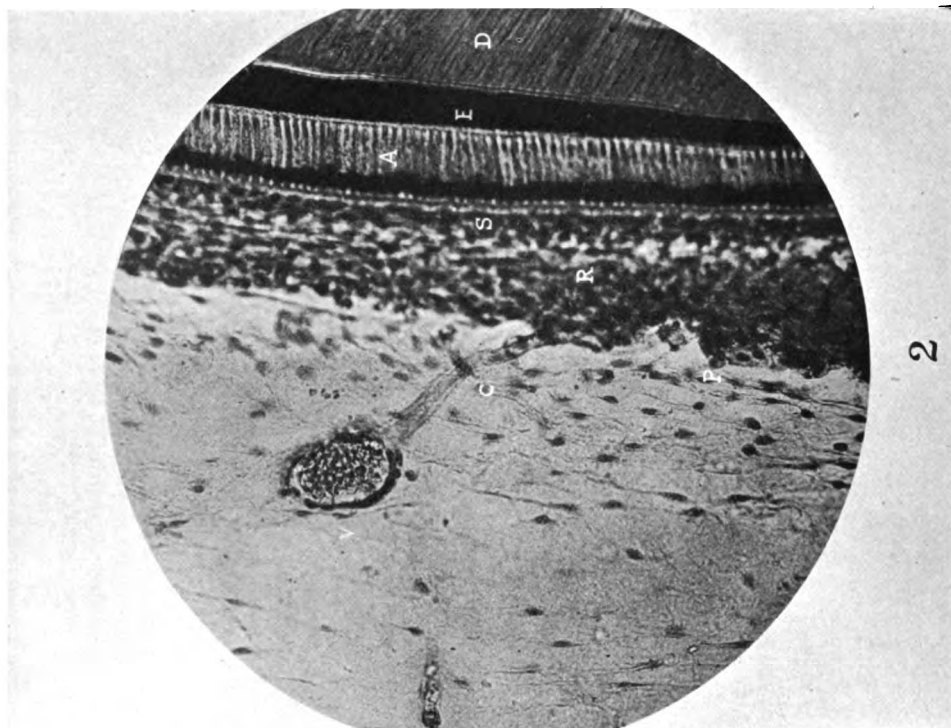


## PLATE 1

## EXPLANATION OF FIGURES

1 Photomicrograph of axial section of lateral incisor of a week-old kitten. The connective tissue of the extensive dental sac is of loose texture. At *V*, in the dental sac, is seen a cross-cut arteriole. A capillary branch, *c*, passes from this arteriole towards the enamel organ, and as it courses along its surface produces a series of indentations with intervening 'papillae,' *p*, the so-called 'papillary layer' of Williams. At the same level, on the opposite surface of the tooth, are shown a similar series of papillae. The stellate reticulum, *R*, is of considerable width. It is bounded externally by a dark line, the outer enamel epithelium; internally by a narrow, more condensed area, the stratum intermedium. *a*, layer of ameloblasts; *D*, dentin. The darker band between the ameloblasts and the dentin is young enamel. *X*, contraction artifact within the stellate reticulum of the coronal apex; *O*, layer of odontoblasts, enveloping the dental papilla.  $\times 40$ . (Photos by William S. Dunn, Cornell University Medical College, New York City.)

2 More highly magnified photomicrograph of the arteriole and the adjacent portions of dental sac and tooth wall shown in figure 1. *V*, arteriole; *c*, a capillary branch. The latter can be traced along the border of the enamel organ to the point, *p*, where is located a large papilla of the papillary layer. *R*, stellate reticulum of the enamel pulp; *S*, stratum intermedium; *A*, layer of ameloblasts; *E*, enamel; *D*, dentin.  $\times 180$ .

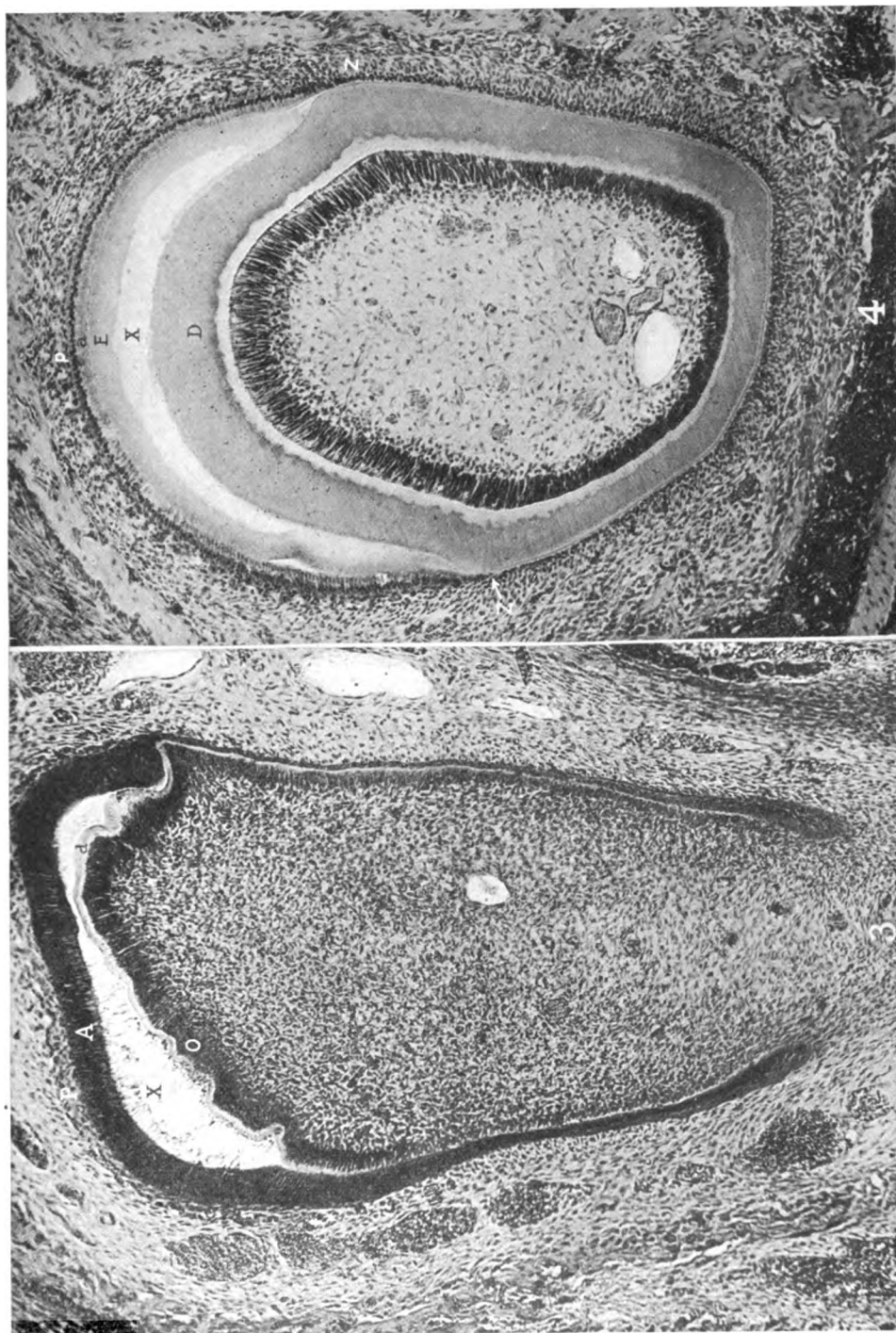


## PLATE 2

### EXPLANATION OF FIGURES

3 Photomicrograph of an axial (frontal) section through the 'root' (base) of the upper incisor tooth of a week-old white rat. On account of the extreme curvature of this tooth (as described in the text), a transverse section of the head cuts the outer border of the tooth above, not the coronal apex as in the case of the kitten's tooth of figure 1. Transverse sections of the head, more anteriorly, yield approximately transverse sections of the tooth, similar to figure 4, where the enamel organ appears only on one surface (the outer) of the tooth. Note the more compact character, narrower width, and more extensively vascularized condition of the dental sac as compared with the tooth of the kitten (fig. 1). Note also the absence of a stellate reticulum in the enamel organ. *O*, layer of odontoblasts, enveloping the dental pulp of the persistent dental papilla; *d*, thin deposit of dentin; *X*, contraction artifact spanned by drawn-out Tomes' processes; *A*, layer of ameloblasts; *p*, papillary layer. The membrane, *p*, constitutes practically the entire extra-ameloblastic portion of the enamel organ. Only a barely perceptible layer of enamel occurs as yet on the outer border of the tooth at this level.  $\times 75$ .

4 Photomicrograph of a slightly oblique transverse section of the lower incisor of a week-old white rat. The enamel organ (*p+a*) is limited to the outer surface. It ends abruptly at points *Z*. Here the layer of ameloblasts, *a*, turns back abruptly into a single layer of low cuboidal cells continuous with the cells of the outer enamel epithelium. Some distance above the points *Z*, the enamel organ becomes several layers thick (three to six layers), and is impinged upon by numerous capillary loops which indent the thin enamel organ and throw it into a series of alternating irregular grooves and elevations, producing thus the papillary layer, *p*. Note the compact character of the connective tissue of the narrow dental sac. *E*, enamel; *X*, fixation (contraction) artifact; *D*, dentin; *O*, layer of odontoblasts enveloping the very vascular dental pulp.  $\times 100$ .

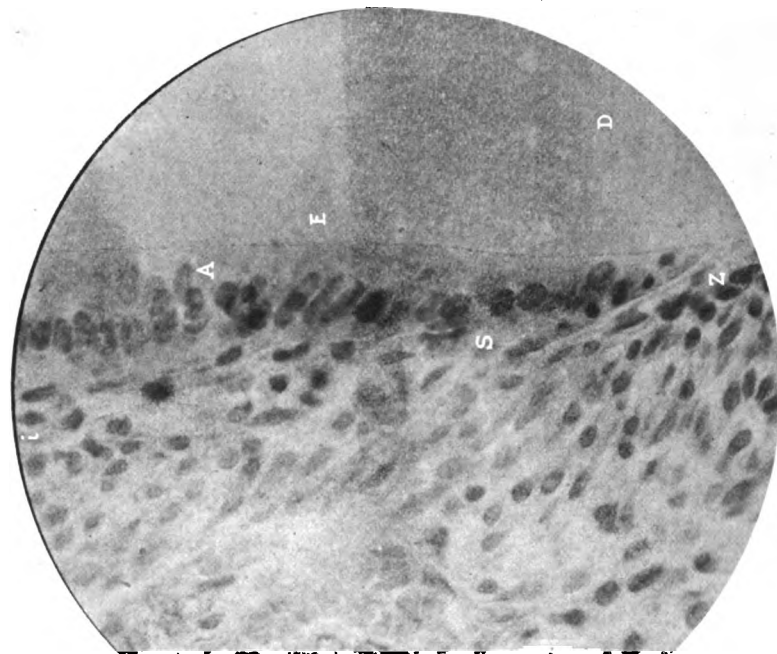


### PLATE 3

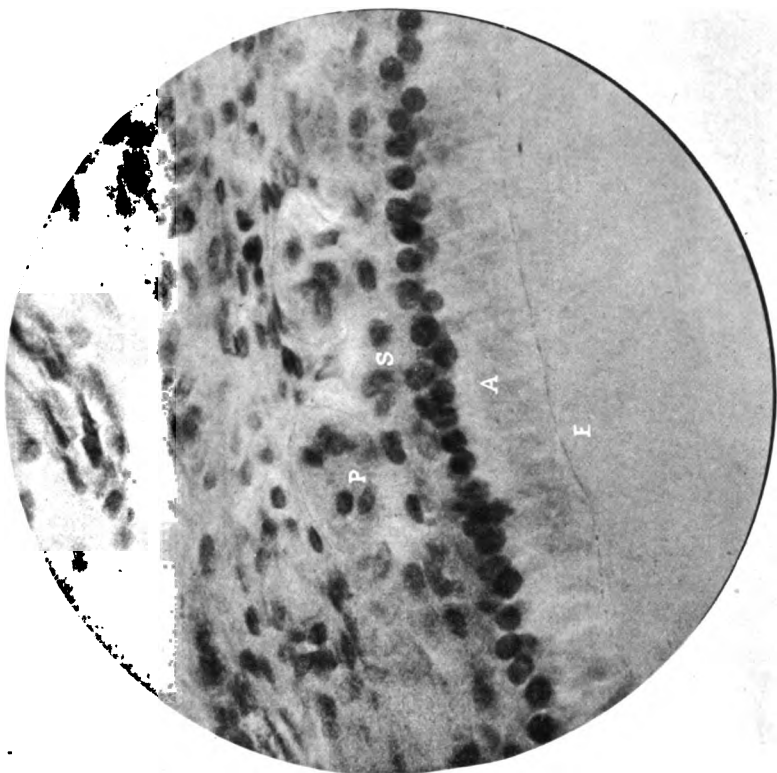
#### EXPLANATION OF FIGURES

5 Enlarged photomicrograph of area *Z* of figure 4. *Z*, point where the layer of ameloblasts, *A*, turns back upon itself into a layer of cuboidal cells continuous with the outer enamel epithelium, *i*. Immediately below *i* is a large capillary; the area between the capillary and the nucleated bases of the ameloblasts constitutes the entire extra-ameloblastic area of the enamel organ, and consists essentially of one or several layers of cuboidal or ovoid cells, the stratum intermedium, *S*. A stellate reticulum is lacking. In the slides, the enamel organ, due to its differential staining reaction, contrasts very much more sharply than shown in the photographs with the outlying connective tissue at the point *Z*. With the hematoxylin-eosin combination of dyes the enamel organ stains a deep pink color, the surrounding tissue of the dental sac takes a light blue color.  $\times 470$ .

6 Photomicrograph under higher magnification of the area *p* of figure 4. *E*, enamel; *A*, layer of ameloblasts; *S*, stratum intermedium, a single layer of cuboidal or ovoid cells; *p*, a papilla of the so-called 'papillary layer.' Between this papilla and the one next on the right is shown a capillary loop. The papillae are covered by the outer enamel epithelium, and involve the entire extra-ameloblastic portion of the enamel organ. Between the papilla, *p*, and the bone, *B*, is a narrow envelope of very cellular connective tissue of this portion of the dental sac.  $\times 570$ .



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Resumen por el autor, Ralph F. Shaner

El desarrollo de la faringe y arcos aórticos de la tortuga, con una nota sobre el quinto arco y el arco pulmonar de los mamíferos.

La faringe de la tortuga produce cinco bolsas faríngeas y un cuerpo postbranquial. La primera bolsa se transforma en el tubo auditivo y la membrana que tapiza interiormente la cavidad tímpano-mastoidea. La segunda desaparece. La tercera produce divertículos dorsal y ventral, los cuales se transforman en el adulto en el timo anterior y la glándula paratiroidea anterior, respectivamente. De un modo semejante se desarrollan a expensas de la cuarta bolsa un timo postero-dorsal y una glándula paratiroidea ventro-posterior. La quinta bolsa se atrofia. El cuerpo postbranquial se pone en contacto con la cuarta bolsa soldándose a ella. En el lado izquierdo se disocia en un grupo de vesículas secretoras y en el derecho degenera. Existen seis arcos aórticos, los cuales aparecen serialmente de delante a atrás. El quinto arco es por completo independiente antes de que aparezca el sexto. El sexto está situado lateralmente al cuerpo postbranquial y comienza y termina en el quinto arco. Cuando este último degenera, el sexto se apropia de sus porciones proximal y distal para formar el arco pulmonar compuesto. A causa de estar el quinto arco de los mamíferos más proximalmente relacionado con el cuarto, apareciendo después del pulmonar, el autor, de acuerdo con Lewis, considera al quinto arco de los mamíferos como un vaso atípico, aunque es probablemente un arco verdadero. Además, puesto que en los mamíferos el arco pulmonar está situado medialmente al cuerpo postbranquial y sigue el curso, no del sexto arco de los reptiles, sino de un vaso irregular transitorio que se encuentra en algunos embriones de tortuga, no puede estrictamente relacionarse con el sexto arco de los vertebrados inferiores.

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## THE DEVELOPMENT OF THE PHARYNX AND AORTIC ARCHES OF THE TURTLE, WITH A NOTE ON THE FIFTH AND PULMONARY ARCHES OF MAMMALS

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FOUR PLATES (FIFTEEN FIGURES)

Although there have been several studies of the development of the reptilian pharynx, few of them have been made with the aid of wax models and the other more exact methods so often used in researches on mammals. This communication embodies the results of an application of such methods to the most available American reptiles, the common fresh-water painted turtles, *Chrysemys marginata* and *C. picta*. Attention has been directed chiefly to the third, fourth, and fifth pouches and the postbranchial body. It seemed worth while to include also a study of the aortic arches. Of the many papers on these vessels, few treat of the relations of the arches to the pharynx. A study of the vessels in connection with the pharynx brings some new facts to light which have a bearing on the nature of the fifth and pulmonary arches of mammals.<sup>1</sup> For the drawings of the models I am indebted to Mr. H. F. Aitken, who has represented these difficult objects with skillful fidelity.

In the turtle, as in reptiles generally, there develop five pouches, exclusive of the postbranchial body. The first four are ordinary lateral outgrowths of the entoderm (figs. 1 to 4). The thin membrane that bars communication between the pouch and the exterior is broken through in the first three pouches; the fourth, in all likelihood, is always closed. Each pouch is furnished with a branchial placode. In younger embryos—up

<sup>1</sup> A preliminary report of these observations was presented at the meeting of the American Association of Anatomists in Philadelphia, 1921. See *Proceedings, Anat. Rec.*, vol. 21.

to 7 mm.—the pouches are deep and fish-like, each with a very narrow slit-shaped lumen.\*

Behind the fourth pouch, in the angle between it and the pharynx, there develops a diverticulum of a different nature. It appears first in a 5.5-mm. embryo (figs. 3, 4), and is pointed more caudally and medially than the true pouch outgrowths. In a 5.6-mm. embryo (fig. 5), it ends in a flattened expanded knob of considerable size. The diverticulum is clearly a postbranchial body.

On the postbranchial body there now appears a secondary outgrowth (fig. 6), which points due laterally and which fuses with a slight indentation of the ectoderm. A branchial placode is placed behind the outgrowth, and the fifth and six aortic arches lie before and behind it, respectively. In short, the secondary outgrowth is a true fifth pouch, which differs from the four anterior ones in arising after the formation of the postbranchial body, and in growing from this body rather than from the pharynx proper.

Such a full development of the fifth pouch and the postbranchial body enables one to make at least a partial interpretation of the postbranchial body. It is not a pouch, for a pouch develops from it: it never has those relations to nerves, branchial placodes, and aortic arches which characterize a true pouch. Neither is the postbranchial body an outgrowth of a pouch, in the sense that the thymus and parathyreoid glands are, for it grows out directly from the pharyngeal wall, and precedes the fifth pouch, the only one from which it could be derived. The true pouch derivatives do not appear until much later, and when they do, the postbranchial body is not in series with them. The postbranchial body is better considered as a caudal prolongation of the pouch-forming area—a remnant out of which the fifth pouch develops, and from which a sixth would spring, if such were to appear.

\* The length of the embryos used in this paper is the head-breech dimension. But turtle embryos coil up at very uneven rates, so that such measurements give only an approximate idea of their age.

Before the various pharyngeal derivatives develop, there are several changes in the general relations of the pouches. The second, third, and fourth clefts come to end in a deep cervical sinus, into which the second and third pouches open and to which the fourth is joined by a solid stalk (fig. 8). The whole embryo grows in such a way that the pouches are first pulled out into long tubes. Next, the clefts are deepened into tubular prolongations of the pouches and the placodes are carried from the external body surface into the recesses of the clefts. Finally the external apertures of the second and fourth clefts are drawn towards that of the third, and all three then are buried in a common cervical sinus.

At the same time the pharyngeal wall between the fourth pouch and the postbranchial body is pulled out into a common stalk for both (fig. 7). The fourth pouch is much the larger. When the common stalk is completed, it appears to be part of the fourth pouch, to which the postbranchial body then seems to be attached (fig. 8).

The fifth pouch meanwhile degenerates rapidly; it is represented in the 9.5-mm. embryo (fig. 8) by a slight heap of cells placed at the union of the postbranchial body and the fourth pouch. In older embryos it is lost altogether.

In the following paragraphs, the several pouches and other pharyngeal structures will be considered in further detail, each in turn.

*First and second pouches.* The later development of the first two pouches can be passed over briefly. From the first there develops, in a 9.5-mm. embryo, a tympanic process. At 10.6 mm., this process pushes forward beneath the columella and then turns back over its dorsal surface. The tip of the process expands into a large irregular sac, which fills the tympanic and mastoid cavities. The proximal end of the tympanic process and the remnant of the first pouch are converted into a relatively large auditory tube, to which the second pouch may also contribute. The method of development of the tympanic cavity is very similar to that of *Chelydra*, which has been carefully described and excellently illustrated by Dohrer.

The second pouch of the 9.5-mm. embryo bears a slight dorsal knob-like outgrowth, apparently the structure described by Van Bemmelen in 1893. It does not appear in younger or older embryos. One is tempted to call it a transient thymus II. It has, however, none of the characteristics of other thymic outgrowths of the same size, and must therefore be considered as of doubtful nature. Both the bud and the pouch disappear in a 10.6-mm. embryo, in which nothing remains of the pouch save a slight furrow on the pharyngeal wall. The furrow may be incorporated in the auditory tube; but if so, it has no effect on the final contour of the tube.

*Third pouch.* On the third pouch, in embryos of around 9 mm., there appear two outgrowths, a dorsal thymus III and a ventral parathyreoid III. The dorsal outgrowth is preceded by a slight crescentic invagination which, in a 6.6-mm. embryo, is placed just medial to the branchial placode. A similar crescent is found in snakes by Saint-Remy and Prenant. In an 8.8-mm. embryo, the crescent is lifted up as part of the regular thymic outgrowth, and there is produced a hollow, indented, lobulated finger, such as is found in a 9.5-mm. embryo (fig. 8).

The ventral outgrowth, the parathyreoid III, develops at the same time. In the 9.5-mm. embryo (fig. 8), it is an undifferentiated, smooth-walled, sac-like recess.

The connections of the third pouch, first with the cervical sinus and then with the pharynx proper, become now broken, so that in a 10.6-mm. embryo (fig. 9) the thymic and parathyreoid outgrowths, with the intermediate pouch-tissue, form an independent complex which lies free in the surrounding mesenchyma, just behind the internal carotid artery.

The dorsal thymic outgrowth grows very rapidly. Its central cavity disappears; the intermediate pouch-tissue is absorbed; and the parathyreoid gland is completely surrounded by the downward extending lobules of the thymus. In a 16.8-mm. embryo (Harvard Embryological Collection, 1090) the cells of the thymus take on the lymphoid appearance typical of thymic tissue. In a 32-mm. embryo (H. E. C. 1127) the lobules show subdivision into cortex and medulla, and within the latter appear thymic corpuscles.

The ventral part of the complex, the parathyreoid III, increases in size much more slowly. Its internal cavity can still be found in a 16.8-mm. embryo, in which it is lined by cuboidal epithelium. The substance of the parathyreoid gland is then invaded by vascular and connective tissue, and the endodermal cells are rearranged into solid cords. In a 27mm. embryo (H. E. C. 1096), the central cavity is lost, and the parathyreoid gland is like the adult organ in all respects.

The two derivatives of the third pouch are present regularly in the adult *Chrysemys picta* as a large anterior thymus (fig. 14), placed at the union of the internal carotid and the subclavian arteries, and, within it, the anterior parathyreoid. The thymus is divided into many lobules bound together by dense connective tissue. Each lobule is easily divisible into a dense cortex and loose medulla. Within the medullary substance occur large irregular thymic corpuscles. Both the medulla and the corpuscles are tinged red with large eosinophilic cells, the protoplasm of which is filled with coarse and brilliant staining granules.

The anterior parathyreoid gland is a small round body made up of solid cords of irregular epithelial cells. The cords are surrounded by a rich network of sinusoids and only a small amount of connective tissue.

*Fourth pouch.* Two outgrowths arise also from the fourth pouch. The small dorsal thymus is preceded by a slight crescentic invagination, less well-defined than in the third pouch, but nevertheless identifiable in an 8.8-mm. embryo (H. E. C. 1086). The crescent is lost at once, and a small solid outgrowth is formed, such as appears in the 9.5-mm. embryo (fig. 8). The ventral diverticulum, the parathyreoid IV, is even larger in the 9.5-mm. embryo than the corresponding outgrowth of the third pouch, with which it is identical in structure.

The stalk connecting the fourth pouch with the cervical sinus, and the common neck by which the fourth pouch and the postbranchial body are joined to the pharynx proper, are next destroyed, so that in a 10.6-mm. embryo (fig. 10) the two outgrowths, with the intermediate pouch-tissue and the attached

postbranchial body, form another independent complex, placed just behind the systemic aortic arch.

The dorsal thymic outgrowth, the thymus IV, undergoes a transformation similar to that of thymus III, save that the thymus of the fourth pouch is always very small and never envelops the parathyreoid gland. The thymus IV apparently absorbs the intermediate pouch-tissue.

The ventral outgrowth, the parathyreoid IV, can be followed in older embryos as a large organ, as large as the corresponding outgrowth of the third pouch. The changes in structure of both are quite similar.

*Fifth pouch.* The transient fifth pouch, at the height of its development, displays a slight crescentic invagination (8.4 mm., H. E. C. 1078), which can be interpreted as a rudimentary thymus V. The whole pouch, thymus and all, then degenerates. It may be represented in the 9.5-mm. embryo (fig. 8) by a heap of cells placed at the point where the postbranchial body joins the fourth pouch. No later traces of the pouch or thymus can be found.

*Postbranchial body.* The postbranchial body at first develops equally well on both sides of the embryo. In the 9.5-mm. embryo (fig. 8), the organ is a large hollow flask with a very narrow neck. When the fourth pouch loses connection with the cervical sinus and the pharynx proper, the postbranchial body is carried away with it to form a part of the complex already referred to (fig. 10). The right postbranchial body now degenerates. Traces of it can be found in older embryos and in the adult. The left body increases rapidly in size and becomes studded with epithelial buds (fig. 10). In older embryos the buds grow at the expense of the original sac, which in a 32-mm. embryo (H. E. C. 1127) is reduced to a group of isolated epithelial vesicles.

The thymus IV, the parathyreoid IV, and the postbranchial body develop throughout embryonic life as a closely associated group of organs. All three can be found in the adult as components of the 'aortic body' of Van Bemmelen ('88), situated along the systemic aortic arch just above the ductus arteriosus. A section of this body is shown in figure 15. The thymus IV

is present as a small posterior thymus, made up of one or two tiny lobules. Each lobule is divided into cortex and medulla, the latter containing the characteristic thymic corpuscles. The parathyreoid IV appears as the posterior parathyreoid gland. In structure it is similar to the anterior gland, save that the posterior one has the more irregular cords, larger sinusoids, and a generally looser texture. The postbranchial body is represented, in the left aortic body, by a great group of spherical vesicles surrounded by a mass of darkly nucleated cells. Most of the vesicles are small and lined with low columnar epithelium. A few are quite large and made of low and very regular cuboidal cells. Many of the small and all of the large vesicles contain a definite reddish-staining secretion. The larger vesicles resemble greatly those of the thyreoid gland. The right postbranchial body is represented in the adult by a few scattered clumps of cells in the right aortic body.

*Thyreoid gland.* The thyreoid gland is a midventral outgrowth of the floor of the pharynx between the first and second pouches. It is present in a 4.6-mm. embryo (fig. 2). In a 6-mm. specimen the gland is still attached to the pharynx by a slender pedicle: in older embryos it is a free flattened mass placed just ventral to the truncus arteriosus and the beginnings of the great vessels leaving the heart. In a 9.5-mm. embryo the cells are arranged in cords, some of which contain very tiny lumina. In a 10.6-mm. embryo lumina are definite; at 16.8-mm. they are large and contain a reddish-staining secretion. The gland retains its embryonic shape and relation to the great vessels throughout life. It never receives any additions from the postbranchial bodies.

*General considerations.* The previous work on the development of the pharynx of the turtle is comprehended in the papers of Van Bemmelen ('86, '88, '93), C. E. Johnson ('19), and von Alten ('14, '16). Van Bemmelen finds five pouches. From the first develops the tympanic cavity; the second bears a dorsal epithelial bud which disappears with it. The third pouch swells out into a large follicle that becomes the (anterior) thymus and the enclosed epithelial body (parathyreoid gland). The



fourth and fifth pouches and the postbranchial body form an independent complex of three closely associated vesicles which retain their epithelial character in later stages. My results are essentially an amplification of Van Bemmelen's—a closer analysis of the complexes arising from the third and fourth pouches. The fifth pouch does not enter into the second complex, as Van Bemmelen thought; the three vesicles composing it are the dorsal and ventral outgrowths of the fourth pouch and the postbranchial body. Johnson, in a very brief abstract, notes the finding of an anterior thymus and anterior parathyreoid gland derived from the third pouch. He finds, however, no posterior parathyreoid, and derives the posterior thymus from the fifth pouch. Without knowledge fuller than an abstract affords, it is difficult to determine the cause for Johnson's radical difference from the results of others, including my own. The suppression of the fourth pouch and the full development of the third and fifth would be a very unusual procedure. The fragmentary report of Von Alten's work, given out by Keibel after his pupil's untimely death, does not extend beyond the earliest stages. Von Alten finds five pouches, notes the branchial placodes, and is inclined to consider the postbranchial body a rudimentary fifth pouch. The last is, of course, a very debatable question. I have given my reasons for rejecting this interpretation elsewhere.

No one writer has given a complete account of the branchial derivatives in the adult turtle. Van Bemmelen ('88) found the adult anterior thymus and enclosed parathyreoid gland, and discovered the aortic body. Beyond noting the epithelial character of the last, he gives no further details. Afanassiew had previously described the anterior thymus and enclosed 'carotid body,' but overlooked the aortic body. On the other hand, Thompson gives a very complete and well-illustrated account of the aortic body in several species. She is the first to recognize that, although a parathyreoid gland and postbranchial body are the chief constituents of the aortic body, a small thymus is generally present. Doyen finds the posterior parathyreoid gland in the 'African tortoise.' His photomicrograph shows

traces of the postbranchial body. Neither Thompson nor Doyen record the much larger anterior thymus and its enclosed parathyreoid. The papers of Van Bemmelen and Thompson are the most complete and supplement each other; my own results are in close accord with their descriptions.

The turtle, in deriving its adult thymus and parathyreoid glands from the third and fourth pouches, and not from the first two, resembles the bird and mammals generally (Verdun). When comparing mammals with other vertebrates, however, one must not forget that the mammalian thymus and parathyreoid gland are ventral and dorsal outgrowths, respectively—an arrangement just the reverse of that found in every other vertebrate phylum. Maurer ('99 a, '06) has pointed out that the thymus III of *Lacerta encroaches* ventrally on to the intermediate pouch-tissue, thus possibly approaching the mammalian condition. The same is certainly true of the thymus III of turtles. There is, however, no change in the position of the parathyreoid gland in either reptile. Maurer ('99 b, '06) emphasizes the absence of colloid in the postbranchial body of all non-mammalian vertebrates, and considers the secretion to appear first in *Echidna*. Whatever may be its chemical nature, a colloid-like secretion is certainly present in the postbranchial body of the turtle—perhaps another of the several adumbrations of the mammalian condition to be found in this reptile.

*Aortic arches.* The aortic arches are six in number. They appear serially from before backward. The first two are transient vessels. The third persists as the internal carotid artery, and the fourth as the systemic aortic arch. The fifth appears soon after the fourth is completed. It becomes a fully developed arch (fig. 6), which extends from the truncus arteriosus to the dorsal aorta, and functions in embryos of from 6.5 mm. to 9 mm. in length. The sixth arch begins as a bud from the fifth, springing from the latter above the fifth pouch (fig. 6) and running ventrally behind the fifth pouch and lateral to the postbranchial body. In the 6.6-mm. embryo the arch ends blindly; in an older embryo of 8.4 mm. (fig. 11), it rejoins the fifth below the pouch and becomes a complete functioning vessel. The sixth arch,

thus constituted, differs from the preceding ones in beginning and ending in the arch anterior to it. For some time the proximal and distal parts of the fifth arch serve as common trunks for the fifth and sixth arches.

The middle segment of the fifth arch now withers away. The proximal and distal segments are then taken over by the sixth arch to form with it the composite pulmonary arch of the 9.5-mm. embryo. The pulmonary arch is not exactly synonymous with the sixth; it is rather the entire sixth arch plus the proximal and distal segments of the vanished fifth.

The pulmonary artery is already present in the 8.4-mm. embryo (fig. 11). It arises from the sixth arch.

In all non-mammalian vertebrates, the fifth and sixth arches are closely related, the two quite generally beginning and ending in a common trunk. Such is the case in birds (Locy), *Lacerta* (Peter), the frog, salamander, and siren (Boas, '82), and *Polyp-terus*, *Ceratodus*, and *Lepidosteus* (Boas, '80). The development of few forms is known in detail sufficient to determine the exact nature of the relation. In *Lepidosteus*, Müller finds the fifth and sixth arches appearing simultaneously from a common ventral trunk. In the frog (Marshall and Bles, '90), the fifth arch is well developed before the sixth appears; the latter begins and quite often ends in the fifth arch in the younger tadpoles. In older tadpoles the fifth arch atrophies, and the sixth, on becoming the pulmocutaneous arch, seems to take over the proximal segment of the fifth arch, much as the pulmonary arch does in the turtle.

Taking non-mammalian vertebrates as a whole, the fifth arch is a vessel which extends from the truncus arteriosus to the dorsal aorta, between the fourth and fifth pouches. It appears after the fourth arch and before the sixth. When the fifth arch is a complete and functioning vessel, the sixth appears, and begins or ends, or both, in the fifth, so that the proximal and distal segments of the latter serve as common trunks for both arches. In reptiles and birds, at least, the middle part of the fifth arch then degenerates, leaving the proximal and distal segments to constitute, with the sixth arch, the pulmonary arch of the older embryos.

In mammals, on the contrary, the so-called fifth arch arises after the pulmonary arch is well established. In many of the reported cases of it, e.g., the original fifth arch of Zimmerman and in many of those of Tandler and Lehmann, the fifth arch is more closely related to the fourth, beginning or ending in the fourth, or both, and otherwise pursuing a highly variable and erratic course. A vessel that appears after the pulmonary arch is completed and has no real relation to it is hardly a fifth arch in the sense that obtains in lower vertebrates.

The position and relation of the sixth arch of the turtle are also of considerable morphological significance. The sixth arch (and the resultant pulmonary arch) lies *lateral* to the postbranchial body (fig. 11, 8.4 mm.). In this particular embryo there is, in addition, a transient vessel (fig. 11, *x*) which arises from the distal common trunk of the fifth and sixth arches, and winds around the medial side of the postbranchial body.

The sixth or pulmonary arches of many other reptiles similarly occupy the lateral position. Such is the case in *Lacerta* (5 mm., H. E. C. 731), *Sphenodon* (7.9 mm., H. E. C. 1491), *Aristelliger*—a geckonid lizard—(4.9 mm. H. E. C. 1884), *Iguana* (10 mm.), and probably in the alligator. Snakes (*Eutaenia* and *Oxybalus*) are possible exceptions; before the relation of their arches can be established, the nature of the posterior part of the pharynx of these forms must be investigated.

In mammals the pulmonary arch lies *medial* to the postbranchial body. The normal mammalian condition is well shown in *Tarsius* (fig. 12 after Hafferl) and in the mole (fig. 13 after Soulié and Bonne). The rabbit and pig are other examples (Lewis).

The postbranchial body of mammals is now generally considered to be a prolongation of the pouch-forming area of the pharynx, out of which a fifth pouch occasionally develops as a lateral outgrowth, just as in the turtle. If the mammalian pulmonary arch is a true sixth arch, it should also lie lateral to the postbranchial body in order to be in proper relation to the fifth pouch. Instead, it lies medial to the postbranchial body and takes the course of the transient branch of the common

trunk of the fifth and sixth arches of the 8.4-mm. turtle (fig. 11) already referred to. This branch is not an aortic arch at all.

What, then, should be the enumeration and description of the aortic arches in mammals? Rathke recognized five arches, formed serially, ending with the pulmonary as the fifth. Boas ('87) 'predicted' and Zimmermann described a new arch between the fourth and pulmonary arches, and embryologists generally thereafter counted the pulmonary arch of mammals as the sixth, while recognizing only four pouches together with the postbranchial body, the nature of which was then uncertain. Subsequently a transient fifth pouch was found, so that the counting of six aortic arches became more consistent, and has been generally adopted.

Before the presence of a fifth pouch had been established, that is, when four pouches and six arches were accepted, Lewis, who considered that the current diagrams of the mammalian arches were unjustified, wrote briefly in protest ('06). He characterized the conclusion in regard to a sixth aortic arch in mammals as "a morphological speculation of much interest," and noted especially that the pulmonary arch is complete before the fifth arch has formed and before the fourth pouch is present, and he indicated in his figures its position medial to the postbranchial body. These findings seem now to be well established, and they show that although physiologically this vessel is comparable with the sixth arch of the turtle, morphologically it is another vessel. We would conclude, therefore, that in mammals instead of designating this vessel as the sixth arch it should be named the pulmonary arch. Correlated with the increasing importance of the lungs, this arch, not strictly homologous with any in the turtle, has developed precociously. Its median position in relation to the postbranchial body removes it from the series of true branchial aortic arches.

Having eliminated this vessel, the comparison between the arches in mammals and turtles becomes very simple. In both groups they are formed in succession from before backwards, in mammals showing merely a reduction in the posterior members of the series, the pouches and their vessels becoming vestigial

together. The mammals, so far as known, have no sixth arch, which should be behind the fifth pouch, lateral to the post-branchial body, and probably beginning and ending in the fifth arch. The fifth arch of mammals is an ephemeral and atypical vessel, not extending from the dorsal to the ventral aorta as in the turtle, but connecting with the fourth arch and showing the variability characteristic of a vestigial structure. Such, at least, are the conclusions suggested by the work of Lewis on rabbit and pig embryos, as compared with the foregoing study of the vessels and pouches in the turtle.

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## PLATES

### ABBREVIATIONS

*I-V*, the five pharyngeal pouches  
*1-6*, the six aortic arches  
*A. p.*, pulmonary artery  
*A. p. a.*, pulmonary arch of mammals  
*C. pb.*, postbranchial body  
*Gl. pth. III, IV*, parathyreoid outgrowths of the third and fourth pouches  
*Gl. th.*, thyreoid gland

*Gl. thy. III, IV*, thymic outgrowths of the third and fourth pouches  
*Hy.*, hypophysis  
*P. d., P. s.*, right and left lung-buds  
*S. c.*, cervical sinus  
*St.*, mouth  
*Tr*, trachea  
*x.*, transient vessel which lies medial to the postbranchial body



## PLATE 1

### EXPLANATION OF FIGURES

1 to 6 Wax models of the pharynx and aortic arches in embryos of *Chrysemys marginata*. Fig. 1, 4.4 mm., H. E. C. 1051; fig. 2, 4.6 mm., H. E. C. 1052; figs. 3 and 4, 5.5 mm., H. E. C. 1057; fig. 5, 5.6 mm., H. E. C. 1060; fig. 6, 6.6 mm., H. E. C. 1069. All figures  $\times 60$ , and are lateral views, except fig. 4, which is a dorsal view.

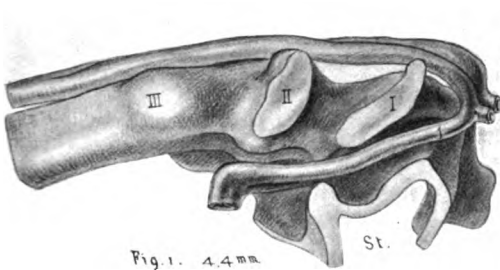


Fig. 1. 4.4 mm.

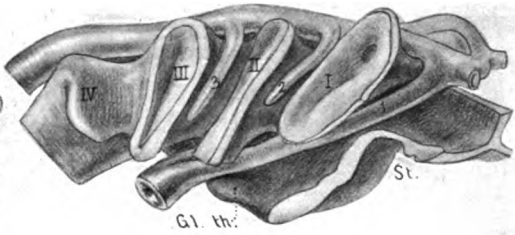


Fig. 2. 4.6 mm.

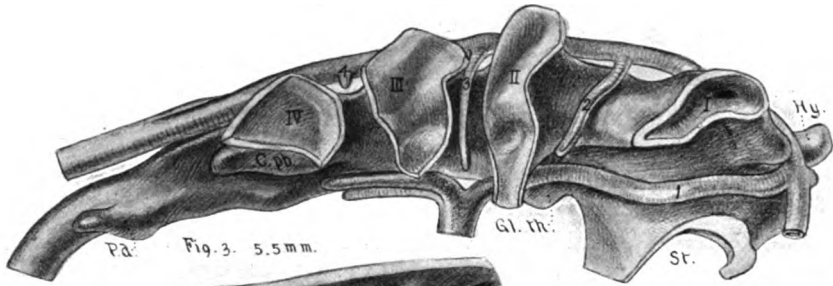


Fig. 3. 5.5 mm.

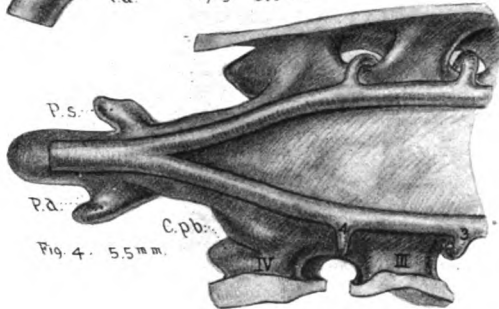


Fig. 4. 5.5 mm.

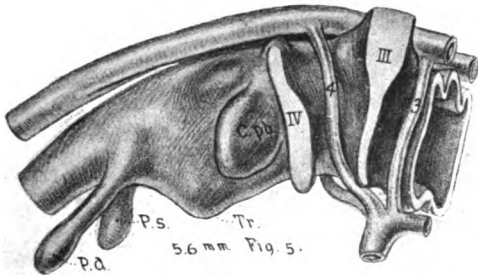


Fig. 5. 5.6 mm.



Fig. 6. 6.6 mm.

## PLATE 2

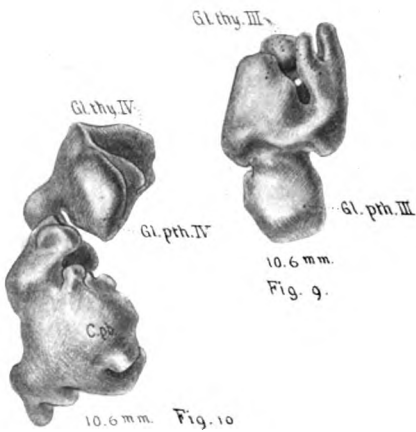
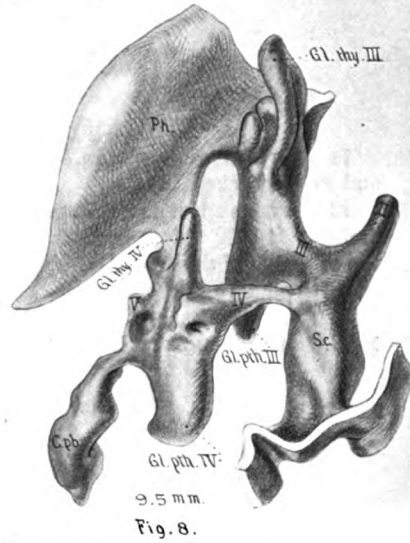
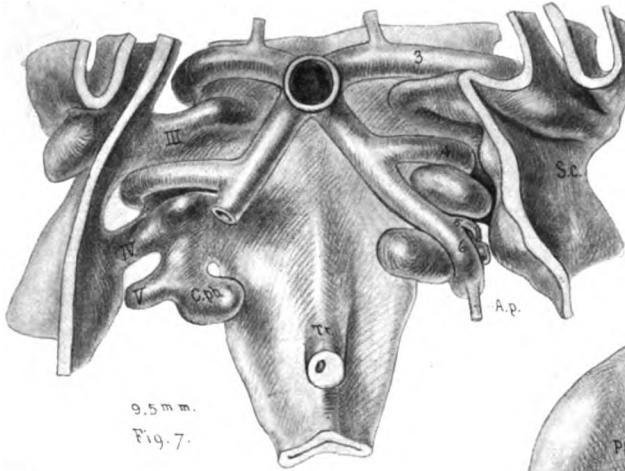
### EXPLANATION OF FIGURES

7 *Chrysemys marginata*, 9.5 mm., H. E. C. 1073. Wax model of the pharynx and aortic arches. Ventral view  $\times 60$ .

8 *Chrysemys picta*, 9.5 mm., H. E. C. 2145. Wax model of the posterior portion of the pharynx, to show the third, fourth, and fifth pouches and the postbranchial body.  $\times 65$ .

9 *Chrysemys marginata*, 10.6 mm., H. E. C. 1087. Wax model of the complex derived from the third pharyngeal pouch.  $\times 60$ .

10 *Chrysemys marginata*, 10.6 mm., H. E. C. 1087. The same embryo as in fig. 9. Wax model of the complex derived from the fourth and fifth pouches and the postbranchial body.  $\times 85$ .



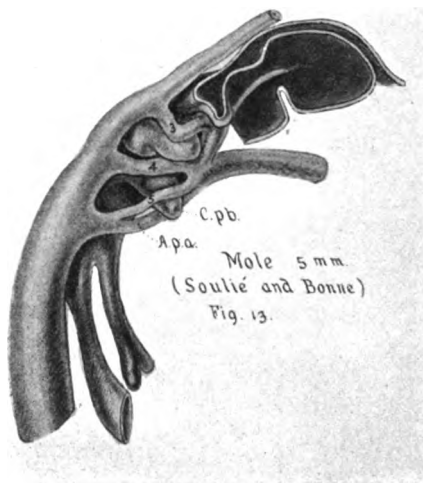
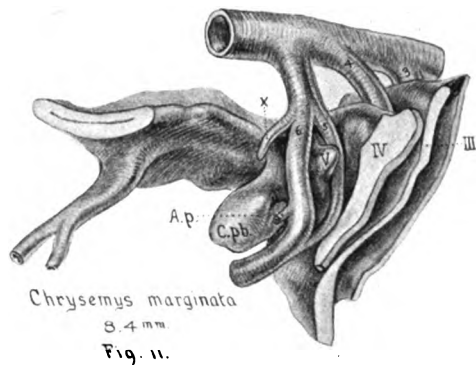
### PLATE 3

#### EXPLANATION OF FIGURES

11 *Chrysemys marginata*, 8.4 mm., H. E. C. 1075. Wax model of the pharynx and aortic arches.  $\times 60$ .

12 *Tarsius spectrum*, 6.7 mm. The pharynx with the aortic and pulmonary arches.  $\times 100$ . After Hafferl. Hafferl's figure has here been reversed. In the originals of this and the following figure, the postbranchial body was named the fifth pouch and the pulmonary arch was labeled the sixth arch.

13 *Talpa europaea*, 5 mm. Wax model of the pharynx and aortic arches.  $\times 38$ . After Soulié and Bonne. Their figure has here been reversed.

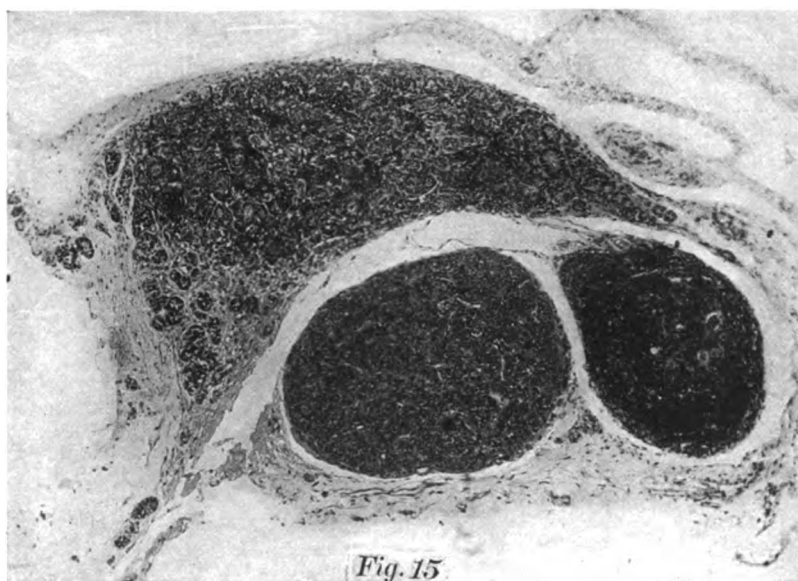
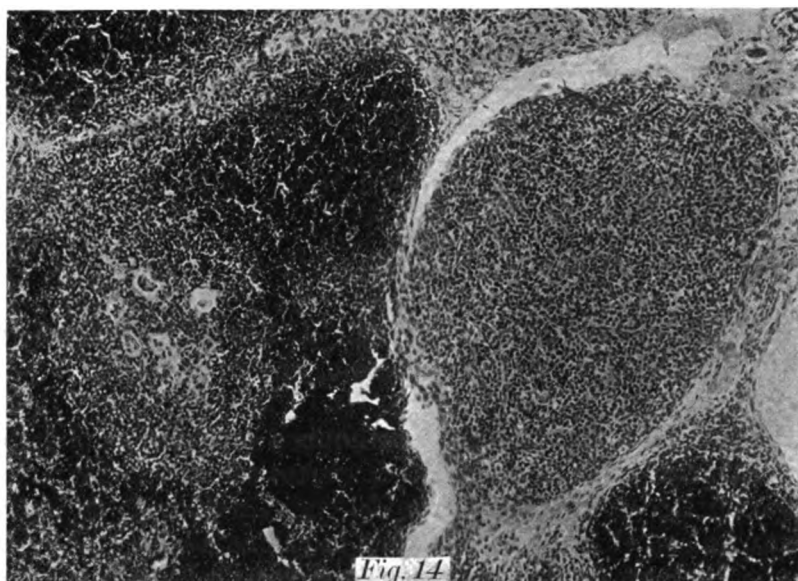


## PLATE 4

### EXPLANATION OF FIGURES

14 Photomicrograph of a section through the anterior thymus (thymus III) and the anterior parathyreoid gland (parathyreoid III) of an adult *Chrysemys picta*. On the left is a part of a thymic lobule, showing dense cortex and lighter medulla, the latter containing thymic corpuscles. On the right is the parathyreoid gland. Haematoxylin and eosin.  $\times 100$ .

15 Photomicrograph of a section through the left aortic body of an adult *Chrysemys picta*, to show the postbranchial body, the posterior thymus (thymus IV), and the posterior parathyreoid gland (parathyreoid IV). The crescent-shaped mass in the upper half of the section is the postbranchial body. Below and on the left is the posterior parathyreoid gland; on the right is the posterior thymus. Haematoxylin and orange G.  $\times 66$ .





Resumen por la autora, Vera Danchakoff

Actividad digestiva del mesenquima. A. Las células del sarcoma de Ehrlich empleadas como objeto de digestión.

La actividad digestiva del mesenquima esplénico es el factor determinante de la desaparición de ciertos tumores de los mamíferos injertados en el alantoides del embrión de pollo en íntimo contacto con el tejido esplénico de la gallina adulta. El tejido de los tumores de los mamíferos (sarcoma de Ehrlich y tumor 180) crece bien si se injerta solamente en el alantoides. Ni el tejido esplénico embrionario ni el mismo tejido muerto y aséptico del adulto afectan su crecimiento. Solamente las células mesenquimatosas vivas del bazo del adulto puestas en contacto inmediato con las células del tumor las rodean y encierran en una cápsula, digiriéndolas después en las pequeñas cavidades así formadas.

Translation by José F. Nonides  
Cornell Medical College, New York

## DIGESTIVE ACTIVITY OF MESENCHYME

### A. THE EHRLICH SARCOMA CELLS AS OBJECT

VERA DANCHAKOFF

*Columbia University, George Crocker Special Research Fund,  
F. C. Wood, Director*

EIGHT PLATES (SEVENTEEN FIGURES)

#### 1. STATEMENT OF THE PROBLEM

Resistance to heteroplastic grafting is a property possessed by a great variety of organisms. Even tumors with their unlimited power of growth cannot be transplanted successfully from one species to another, and transplantation to another animal of the same species has often been followed only by partial success. Among higher vertebrates an entire lack of resistance to certain heterogeneous rapidly growing tumors has been demonstrated in only a few instances. Murphy and Rous grew different tumors on chick embryos. Bullock succeeded in growing mouse sarcoma in new-born rats.

Although various hypotheses as to the factors of resistance have been formulated, none is actually fundamental, though some are apparently well supported. Both organs and cells have been suggested as factors responsible for natural as well as induced resistance against the grafting of tumors. The spleen among organs and the small lymphocytes among cells have been made subjects of special study in this connection. Inhibition of tumor growth due to the presence of the spleen in the organism has been affirmed and denied, as has an inhibiting effect of the splenic tissue on the growth of a tumor graft. On the other hand, receding tumors are often infiltrated by small lymphocytes, and it has been suggested that the presence of these cells may be a factor in the recession.

An attempt was made by Murphy<sup>11, 12, 13, 14</sup> to furnish experimental proof of the significance of the small lymphocytes as factors of natural and induced immunity. On the basis of Murphy's work, Jacques Loeb<sup>10</sup> has suggested that the greater tolerance of plants to grafts of foreign tissue, as compared to animals, may be explained by the absence of 'leucocytes.' Recent work by Bullock,<sup>1</sup> Stevenson,<sup>23, 24</sup> Sittenfield,<sup>20, 21, 22</sup> and Wood and Prime,<sup>26</sup> however, indicate that the rôle of the small lymphocytes in resistance is more than dubious. Further attempts to establish a causal connection between immunity and the presence of 'leucocytes' in general seem, therefore, to be unwarranted.

The mechanism of the resistance attributed to the action of the spleen as a whole or to that of the small lymphocytes has not been discussed, and little, if anything, is known concerning the reason for the failure of a tumor to grow in an immune animal. It is true that a detailed morphological study by Russell<sup>18</sup> and Woglom<sup>25</sup> of the conditions at the site of a graft which has failed to develop has revealed a lack of a stroma reaction in the immune host and a failure of vessels to grow into the grafted tissue which resulted in the subsequent necrosis of the graft. This observation, important as it is, gives no further information concerning the nature of the resistance; the reason why the vessels do not grow into the grafted material in an immune animal remains unexplained.

The purpose of the present paper is to show that the phagocytic and digestive capacity of the mesenchymal syncytium or cellular reticulum of the adult chicken spleen is a direct and decisive factor in the destruction of the Ehrlich mouse sarcoma, and a factor of partial inhibition to the Crocker Fund mouse sarcoma 180, both of which grow vigorously when grafted alone upon the allantois of the chick embryo. The mechanism of this inhibition is easily demonstrable and will be described in detail. I am far from being able to extend my results to other tumors. On the contrary, numerous indications point to the fact that the capacity of the splenic mesenchyme to destroy living tumor cells, as described in this paper, is due to the association of very defi-

nite factors. This activity is, however, a part of the general conditions governing the resistance to heteroplastic tissues, and, as an easily demonstrable phenomenon, may give some clues to the understanding of other manifestations of the resistance of organisms against heteroplastic grafting.

## 2. MATERIAL AND ITS ARRANGEMENT

The loose mesenchyme with its capacity for various differentiation is a site of elaboration of a variety of products either stored in its cellular derivatives or possibly given up into the organism. The introduction of various substances into the organism stimulates the activity of the loose mesenchyme and its cellular derivatives and may be followed even by a modification of mesenchymal differentiation. The activity of the mesenchyme and of its cellular derivatives as a possible factor in the resistance of the organism against heteroplastic grafting has been discussed in some of my previous papers.<sup>3, 5, 6, 7, 8</sup>

The formation of macrophages is of interest in connection with the digestive power of the mesenchyme. Even during the normal course of development groups of blood-corpuscles, for example, may be cut off from the general circulation through the rearrangement of embryonic vessels and numerous blood-cells may be found free within the mesenchyme. Mesenchymal cells are, then, isolated from the common syncytium and develop a phagocytic activity against these stranded elements. This fact alone demonstrates the digestive capacity inherent in the mesenchymal cell in the developmental stage of a macrophage.

Under ordinary conditions, cells do not often come into contact with unmodified proteins. However, mesenchymal cells may take up and digest any small unsplit particles of protein of their own in the form of dead or weakened cells or cellular debris with which they may be in contact. This they accomplish only with the aid of ferments capable of splitting proteins. How far does this power of the mesenchymal cell extend? Does it control heterogeneous proteins and heterogeneous living cells? Is the digestive capacity of the mesenchymal cells an intracellular property only or may they give off their ferments as a secretion like the glandular cells of the endocrine organs?

With these problems in view, I began the study of the mutual relationship of mesenchyme and a fast-growing heterogeneous tissue. An objection to the use of embryonic mesenchymal tissue is found in the fact that the tissues of the embryo, in contradistinction to those of the adult, do not offer resistance against heteroplastic grafting. Therefore, it seems rather improbable that the power of digesting a foreign living cell is an inherent property of the embryonic mesenchyme.

The relation of adult splenic mesenchyme of the fowl to mouse sarcoma has formed the object of my study, and the allantois of the chick has been chosen as a culture medium, in which the activity of the mesenchyme may be easily followed. The loose structure and rich vascularization of the allantois have been shown to offer to the grafted adult splenic mesenchyme of the chick conditions favorable not only for the life of its cells, but also for their further growth and differentiation. A recent observation on the growth of the adult splenic tissue of the fowl on the allantois<sup>4</sup> has shown, moreover, that the phagocytic activity of the grafted mesenchymal cells of the adult spleen against erythrocytes and occasionally against granular leucocytes of the chick may be extensively revealed in this environment.

The presence of the small lymphocytes in the meshes of this tissue is a distinctly unfavorable characteristic of the splenic mesenchyme, especially in a study of the bearing of the mesenchymal activity upon the resistance of the organism against heteroplastic grafting, since the small lymphocyte itself is considered by some investigators to be a factor in this resistance. However, a previous study of the growth of the adult splenic tissue of the chick on the allantois has shown (Danchakoff<sup>4</sup>) that the small lymphocytes are not viable within the allantois. They rapidly emigrate from the transplant into the spaces of the allantois where they are frequently seen to disintegrate and to be ingested by the embryonic mesenchymal cells of this organ. At any rate, their presence in the grafted splenic mesenchyme is always easy to detect and take into account.

In order to determine whether the mesenchymal syncytium of the adult spleen has a power over a living heterogeneous cell, only such cells can be used as give an extensive growth when grafted upon the chick allantois. Rapidly growing transplantable tumors offer the most suitable material. The Ehrlich mouse sarcoma and the Crocker Fund mouse sarcoma 180 were chosen. In comparison with the epithelial tumors and the Flexner-Jobling sarcoma of the rat, both tumors seemed to present great advantages for my purpose. A study of their growth in the allantois has shown them arranged in this environment in the form of a labile syncytium. Their cells are highly mobile and they frequently show protruding processes (fig. 1), and may wander out individually into the chick mesenchyme. This character sufficiently explains the diffuse growth of this tumor along the periphery of the graft, and the infiltration of the loose tissue of the allantois. The same property would tend to effect a closer intergrowth with the splenic mesenchyme, if both tissues were grafted together. Moreover, when occasionally found within the lumen of an eroded vessel, the cells of these tumors are seen to lose all their connections with the common syncytium and to become spherical. Except for size, they are in such a case extremely similar to hemoblasts, but, unlike them, they never show any sign of progressive differentiation.

The tissues for most of my present experiments were crushed by forcing them through a syringe with a sieve bottom. This procedure does not injure many of the cells nor even particles of tissue, but it loosens the texture of the tissue and seems to facilitate the access of cells and groups of cells into the allantois.

Grafted simultaneously and in close contact, both spleen and tumor tissues grew, as already shown by Stevenson. After six to seven days a large growth was usually found in the region of the graft, of which one part consists of splenic and the other of tumor tissue. A detailed analysis of the growth of both tissues at various periods was studied, especially in the region where the tissues come together. This analysis is given in section 4. Both tissues grew well at the periphery, except where they came into contact. Contact occurs at somewhat different

stages after grafting, and a peculiar reaction invariably was developed in this region by the splenic mesenchyme, which strongly reminds one of the phagocytic activity of a macrophage. The phagocyte in this case is not a single cell, however, but a mesenchymal syncytium with a common cytoplasm and numerous nuclei; the object of attack is the living cell of a mammalian tumor. As a result of this reaction the tumor stops growing at the place of contact with the splenic tissue, although it grows well in contact with the loose mesenchyme of the allantois.

Is this mesenchymal reaction to be interpreted as a result of a mere local interaction between adult splenic mesenchyme and cells of the Ehrlich sarcoma and is the allantois of the embryo therefore no more than a culture medium? Or does the embryo itself, at whose expense the graft is growing, enter as a factor into the results obtained? The fact alone that the reaction develops only in the strictly limited region in which both tissues come into contact and does not extend to the whole tumor seems rather to make the participation of the host as a factor in this reaction doubtful.

In order to extend the mesenchymal reaction to the whole tumor graft, both tissues were brought into more intimate contact. Crushing splenic and tumor tissues together and mixing them carefully gives an intimate contact of the two kind of cells. A preparation of such a mixture shows small particles of the tumor tissue enveloped by strands of splenic mesenchyme, the latter occasionally appearing in the form of small islands. Such a mixture of both tissues was used as a grafting material and the progress of the growth of these double grafts was followed day by day. This series of experiments has been repeated five times on sixty eggs, with invariably identical results, described in section 5. Moreover, such series of experiments have been repeated through a period of eighteen months on smaller groups of eggs. Though an attempt has been made to ascertain whether the connective-tissue cells in other regions of the body were capable of exercising a similar digestive activity against the Ehrlich sarcoma cell, no definite results have yet been obtained.

It is difficult on the basis of these experiments, to be sure that in the presence of the adult splenic graft the allantoic mesenchymal cells themselves do not begin at least to take part in the described activity. No definite morphological criteria exist which would permit of a sure identification of allantoic and splenic mesenchymal cells, for both are characterized by the same structural features. It is possible, however, to demonstrate by another series of experiments that the embryonic mesenchymal cells are incapable of destroying the tumor by phagocytic activity whether adult splenic tissue is present or not in the host. If tumor tissue and adult splenic tissue are grafted, widely separate, as described in section 6, the growth of the tumor is not affected by the activity of any mesenchymal elements. It is true that the tumor does not always grow as well as if grafted alone, but, as the analysis of the conditions will show, the factor damaging the growth of the tumor in this case is the myeloid metaplasia of the embryonic mesenchyme following a single spleen graft.

The existence of a phagocytic destructive power in the embryonic splenic mesenchyme similar to that revealed by the adult splenic tissue against the living Ehrlich sarcoma cell, though made improbable by the lack of resistance in the embryo, is not, however, altogether precluded. On the other hand, the cells of the embryonic allantois itself might have been stimulated to a similar digestive activity by extracellular substances brought in with the adult splenic tissue. In order to obtain some definite information concerning the phagocytic digestive capacity of the embryonic mesenchyme, two other series of experiments were made and are described in section 7. On the one hand, the Ehrlich sarcoma thoroughly mixed with splenic tissue of embryos up to the hatching period was grafted; on the other, grafts of the Ehrlich sarcoma mixed with aseptic dead splenic tissue only and together with embryonic spleen were made. The tumor grew well in both cases, and no mesenchymal reaction developed around the tumor cells.

It seemed desirable, finally, to approach the question whether the destructive power of the adult splenic mesenchyme of the



chick against the Ehrlich sarcoma cell is a specific reaction strictly limited to the substratum used. Other series of experiments were made, in which other tumors were grafted on the allantois in mixture with adult splenic mesenchyme. Tumor 180, carcinoma 63, and the Jensen rat sarcoma were thoroughly mixed with the splenic tissue of an adult chick and grafted on the allantois. A destructive mesenchymal reaction similar to that against the Ehrlich sarcoma has been observed only against tumor 180. Though similar to that obtained against the Ehrlich sarcoma cells, the reaction against tumor 180 has never been found as pronounced, unless quantities of spleen two or three times greater than the amount of tumor were grafted.

### 3. GROWTH OF THE EHRlich MOUSE SARCOMA IN THE CHICK ALLANTOIS

#### *Changes of the allantois at a late stage of incubation as factor of tumor recession at this time*

The vascular membrane found in the chick egg beneath the egg-shell membrane, commonly called the allantois, consists in fact of two membranes, the serosa and the allantois. The membranes fuse with each other by their mesodermal surfaces and a rich net of allantoic capillaries grows through the ectoderm of the serosa and expands over its surface as the respiratory embryonic organ (Danchakoff<sup>14</sup>). The rich vascular net which, in later stages (after 8 to 9 days of incubation) is in direct contact with the egg-shell membrane, assures the nutrition of the graft.

Growths of Ehrlich sarcoma grafts have been studied from one to thirteen or fourteen days after grafting. Tumors not older than two weeks have been used, in order to avoid as far as possible inclusions of necrotic masses in the grafted material. A microscopic study of tumors at this age has shown, however, that small necrotic areas were frequently present, especially in the center of the tumor. Before grafting the tumor was forced through a syringe with a sieve bottom, the grafted material consisted always of living cells together with an inconsiderable amount of dead cellular material. Small particles of this mash

were deposited on the allantois at seven days of incubation, and two to four eggs opened every twenty-four hours.

Macroscopically, the grafts were found already established within twenty-four hours. They enlarged steadily for eight to nine days. From that time on, no further growth could be demonstrated, and at about two to three days before hatching, the tumor was seen to become opaque and necrotic and to assume a yellowish tint.

The microscopical study of a tumor graft twenty-four hours after grafting shows that, as has been found in the study of the splenic grafts, only that part of the graft survives which is in close touch with the membrane. At the time of the grafting (seven days of incubation) the allantois is covered by an uninterrupted epithelial layer of the ectoderm of the serosa, which rarely offers sufficient mechanical resistance to prevent the take of the graft. Twenty-four hours after grafting, tumor cells adjacent to the allantois are already in contact with the mesodermal constituents, the epithelial covering of the serosa having become interrupted in many places. Tumor cells undergo frequent mitoses, especially along the channels of the rich vascular net. They round up during the process, but during the resting stage they send out numerous long and slender processes into the depth of the allantois. The sudden appearance of the tumor cell does not call forth any appreciable reaction in the allantoic tissue. The grafted tissue having come into contact with the allantois over an area of about 1 to 4 sq.mm., numerous tumor cells find access into the allantois and become the starting-point of a proliferating neoplasm. The graft is seen sometimes to break as a whole through the epithelial layer of the allantois, and both surviving cells and necrotic masses lie within an edematous allantois.

Numerous interesting details are observed in parts of the graft which do not come into immediate contact with the allantoic tissue. Not all of the tumor cells undergo a process of necrosis and disintegration, though many of them do. A number of tumor cells, however, even originally at a certain distance from the vascularized mesenchyme, survive and show themselves

to be necrophages in a high degree. Podwissotski and others have described the process of necrophagism in various tumors; living tumor cells ingest débris of dead tumor cells which inside of the phagocyte have been mistaken for parasites. In the grafts, tumor cells are seen to ingest dead tumor cell material in great quantity. Necrotic tumor cells, of which the structure is still recognizable, as well as cell material which has entirely lost its structure are incorporated into the cytoplasm of tumor cells, fragmented, and finally completely digested.

It is remarkable that though embryonic mesenchymal cells show a great tendency to surround the extravascular erythrocytes or dead cell material of their own kind, they seldom show any inclination to ingest dead tumor cells. It is true that they are very small in comparison with the tumor cell, but they show no tendency to surround them even in the form of plasmodial accumulations or giant-cells. Most of the tumor cells on the contrary are generally seen to contain in their cytoplasm acidophilic necrotic masses which gradually disappear. The tumor cells have to be considered, therefore, as having a positive chemotropism toward their own dead cell material, and to possess an intracellular digestive power over it.

It is interesting to observe the effect of a great quantity of ingested necrotic cell material on tumor cells undergoing mitosis. The mitotic figure may become greatly distorted by the accumulation of the ingested material, and the chromosomes grouped in a very irregular manner. Other interesting consequences may be observed in the division process of a cell with ingested material. If the latter chiefly occupies one pole of the cell, the division process in the cell proceeds as in a meroblastic egg; a complete division fails to take place, though a deep furrow passing through the active cytoplasm indicates the respective boundaries of the two cells. Both of the cells may simultaneously undergo a further mitosis. The presence of large ingested masses may lead in this manner to the production of multipolar mitoses sometimes more or less regular, at other times remarkable for their irregularity.

Dead tumor cells in various stages of disintegration are not the only material which the tumor cells are capable of ingesting.

Chick erythrocytes as well as granulocytes may also be found within their cytoplasm. During grafting the production of a slight traumatic hemorrhage can rarely be avoided and erythrocytes of the host are frequently brought into contact with tumor cells. Small vessels of the host are frequently invaded by tumor cells in later stages of growth; in both cases the tumor cells are seen to contain chick erythrocytes within their cytoplasm and gradually reduce them to small particles of acidophilic material.

Numerous granulocytes are also found in the tumor cells. It is, however, difficult to decide whether the tumor cell has ingested these cells or whether the leucocytes entered into a weakened tumor cell, since the structure of both often remains unchanged. Other pictures seem, however, to indicate that tumor cells are capable of digesting chick leucocytes, and both in the resting stage and in mitosis they may contain leucocytes in various stages of disintegration. It seems, therefore, logical to conclude that the tumor cell has a great ingestive and digestive power over its own dead material and over chick erythrocytes and leucocytes.

The presence of numerous tumor cells acquiring a gigantic size is very characteristic of the Ehrlich sarcoma grown on the chick allantois. A study of the life-history of these giant tumor cells, by means of microscopical preparations, clearly shows that their metabolism becomes gradually modified.

I have already mentioned that the reconstruction of the chromosome complex in tumor cells may be modified by the presence of large quantities of ingested material which offers a physical obstacle to the regular rearrangement of the mitotic figure. In later stages the chromosomes themselves appear strikingly modified in a great number of giant tumor cells. The chromosomes seldom if ever enlarge, but they are frequently seen to undergo the process of fragmentation and gradual reduction. They appear then in the form of very small rods and even dots. The whole amount of chromatin in the form of the entire chromosome complex appears finally reduced. It is remarkable, however, that tumor cells even with greatly modified chromosomal complexes are still capable of division.

It is interesting to observe how resistant the tumor cells show themselves even under unfavorable conditions. Occasionally in a graft the ectodermal epithelial layer of the serosa does not give way, and separates the grafted tissue from the mesodermic part of the serosa and allantois. In the case of a graft of any other tissue, it becomes quickly necrotic; but in the tumor grafts, numerous cells survive for at least four to five days. At a time when the mammalian mesenchyme and the endothelium of the vessels grafted as a part of the tumor are dead (fig. 2) the tumor cells still manifest an intense activity; they are frequently found in mitosis and are seen to ingest dead tumor material and mammalian erythrocytes. It seems, therefore, that the mammalian tumor cells are more readily adapted to rather unfavorable conditions than are other cells of the mammalian soma.

The outer necrotic zone of the graft is gradually reduced by the digestive activity of the tumor cells, and on the fourth or fifth day of growth hardly any of it remains. The process of growth in the first four to five days is very rapid and mitotic figures are observed in great numbers. An illustration of the growth of a tumor in the allantois on the third day is found in figure 1. The group of tumor cells in the upper right corner is adjacent to the central part of the tumor, of which the texture is dense and in which the reciprocal relations of the cells are not easily determined. In the peripheral zone of the tumor, the cells, as represented in figure 1, are arranged in a loose manner and almost all of them are connected by cytoplasmic processes, and appear in the form of syncytia. In the extreme outer zone of the tumor, the cellular syncytium is broken and small groups of tumor cells are found dispersed within the mesodermic layer of the allantois. Here the tumor cells are characterized by long and slender processes which protrude into the loose tissue of the allantois. Many cells are in mitosis, as the figure shows. It is interesting to note the rounding up of the tumor cells during mitosis in connection with the recent findings of Robert Chambers,<sup>2</sup> who has shown that at this stage of the life-history the cytoplasm of the cell becomes liquefied and the cell

assumes a spherical form. The allantoic mesenchyme with vessels is found to be abundant in the outer zone of the tumor and scanty in the more central part of it. Both kinds of tissue, mammalian tumor cells and chick mesenchyme, present a healthy appearance and show no signs of interaction.

Besides the changes brought about by the exercise of its intracellular digestive capacity, the tumor cell grown on the allantois shows other alterations, probably due to the effects of the culture medium. From about the fourth or fifth day after grafting, inclusions appear in the cytoplasm of the tumor cells which resemble crystalline forms. In Zenker-formol fixation and eosin-azur these inclusions appear as clear separate linear or fusiform spaces of various sizes. They may be curved in adaptation to the curvature of the nucleus or to that of the cell periphery. These uniform spaces were probably occupied during cell life by a formed substance somewhat flexible in its consistence. The number of the inclusions in one cell may greatly vary, and they are present during both the resting stage of the cell and mitosis. Sometimes they may be seen between the chromosomes of the equatorial plate keeping them apart, but are never found within the nucleus so long as the nuclear membrane is intact. The inclusions must be interpreted as due to a change in the metabolism of the tumor cells grown upon the allantois, since those cells in their original host in the mouse do not present such inclusions. Great differences in size and modified nuclear-cytoplasmic proportions are further characteristics of tumor cells growing on the allantois. There is an interesting correlation between the size of the tumor cells and the basophilia of their cytoplasm, the smaller cells being much more basophilic during the resting stage. This seems to correspond with the conception of the cytoplasm as at least a partial center of synthesis of chromatic material for the further building up of the chromosome complex. The highly chromatic nucleolus is also very characteristic of the tumor cell in its resting stage. During the spireme stage of mitosis numerous chromosomes seem to be firmly attached to the nucleolus, which gradually loses its basophilic character as the chromosomes

arise, suggesting that a part of the chromatic material might be furnished by the nucleolus. These changes of the tumor cells within the allantois are especially suggestive in connection with the work of Stevenson,<sup>23, 24</sup> who found a general lowering of the vitality of tumors when grown on chick embryos for many generations.

The tumor grows in the above manner until about the seventeenth to eighteenth day of incubation, i.e., for ten to eleven days and it may attain a diameter of 1 cm. and often reaches 5 to 8 mm. in thickness. Occasionally tumor cells penetrate vessels and small hemorrhages are found within the tumor; the tumor cells here exhibit their ingestive and digestive power. Hemorrhages are usually followed by leucocytic infiltration and some of the granular leucocytes may at the same time infiltrate parts of the tumor. Leucocytic infiltration in a single tumor graft is not a regular occurrence; it frequently takes place, however, in the peripheral parts of the tumor, where a greater number of vessels is present, especially in later stages.

The growth of the tumor regularly stops at about the seventeenth day of incubation. Murphy believed that the development of small lymphocytes in the host at this time furnished a defensive mechanism against the further growth of a heteroplastic tissue. But curiously enough, no small lymphocytic infiltration can be found around the growing graft at this stage.

While the tumor recedes necrotic zones appear usually in the midst of the tumor tissue and gradually extend over larger areas without marked reaction around them. No more indication for a causal connection between tumor recession and the development of small lymphocytes exists than for a connection with any other aspect of the embryonic histogenetic processes.

A thorough analysis of the changes in the allantois itself at this period of development is important in connection with the regression of the tumor. These changes affect not only a heterogeneous graft, but equally the growth of any graft, even that of embryonal chick tissue, such as kidney, testis, and spleen, which gradually become necrotic at this time.

*Changes in the allantois during the last three days of incubation*

Regressive changes are found in the vascular net of the allantois about the eighteenth day of incubation, and on the nineteenth day parts of the capillary network extending over the serosa are found collapsed or disappeared altogether over large areas. The ectoderm of the serosa, which at definite stages occupied a deeper position in the membrane, now again becomes superficial. A curious vacuolization of numerous ectodermal cells is observed and the epithelial layer of the serosa becomes thickened.

Conspicuous changes develop in the walls of the vessels, especially of the arteries. In the earlier stages the walls are very thin with hardly perceptible muscular coats, while in the latter stages of incubation they become thick with heavy layers of muscular tissue. The partial destruction of the superficial capillary net might well explain the hypertrophy of the muscular coat of the arteries and a consequent increase of the blood-pressure in them. Another immediate consequence of the destruction of the capillary net is the development of an edema through the allantois, which in the last days of incubation is many times thicker than in earlier stages. Its mesodermal tissue contains few cells and the vascular channels are widely separated. The edema, a natural consequence of the destruction of the capillary net, itself becomes a factor in the further destruction of this net. These changes in the allantois coincide with the arrest of growth in the graft and are of a character to suggest an inhibiting cause.

Striking changes are frequently observed in the vascular net of the graft itself. Greatly distended capillaries are seen to be engorged with stagnating blood (figs. 3 and 4). The red corpuscles lose their characteristic structure, their nuclei disintegrate and are no longer discernible. Sometimes they fragment; in other cases they flow together and form large masses of a substance showing the staining reaction of hemoglobin. The endothelial walls of the vessels break down, individual endothelial cells begin to show phagocytic activity and ingest the huge masses of protein substance so formed. Red blood corpuscles reduced to



structureless masses along with unchanged erythrocytes are also found in the interior of the greatly distended macrophages. This process is illustrated by figures 3 and 4. It extends gradually to larger vessels around the grafts and numerous vascular channels are seen to be distended by these peculiar thrombi.

This process of occlusion of the vascular net is followed by necrosis of the previously healthy tissue of the graft. In some cases, however, parts of the graft become necrotic without the described changes having occurred. Microscopic study shows in this case a gradual passage of the necrotic focus into the surrounding healthy tissue without appreciable reaction around it.

Heteroplastic tumor as well as homoplastic tissue grafts are equally affected at seventeen to eighteen days of incubation. While the allantois forms a favorable culture medium at the time of the grafting, it gradually and progressively becomes less and less so. These local changes in the milieu, and not the development of a particular kind of small lymphocyte at a distant point from the tumor growth, seem to bring about the recession of the tumor, as well as that of homogeneous grafts. Even anlagen of organs of the same age as the host grafted on the allantois at the seventh to eighth day of incubation begin to become necrotic at about the eighteenth day. There is a correlation between the size attained by the graft and the day of incubation at which the graft becomes necrotic, larger grafts becoming necrotic earlier than smaller ones; a small graft may still find sufficient nourishment even in a modified allantois.

#### 4. GROWTH RESTRAINT OF A TUMOR IN DOUBLE ADJACENT GRAFTS OF TUMOR AND ADULT SPLEEN

The double grafts of tumor and spleen were made on the allantois with small separate quantities of tumor and spleen mash, practically touching each other. Microscopically, the growing grafts were only seldom found to be in contact during early stages. This is accounted for by the fact that the growing points of the tumor and spleen may not necessarily be situated at the place where the tissues come together. The allantoic mesenchyme which separates the two tissues in the first days of

their growth is seldom found to be more than a narrow strand, and usually both tissues come together on the fourth to the sixth day after grafting. There is little to add to the description of the inauguration of tumor growth given in the preceding section; the proliferation of the tissue proceeds in the proximity of an adult spleen graft in the first days after grafting in the same way as does the single tumor graft.

An inhibition of the growth of the tumor graft usually does not begin until the two grafts have come together; but in cases in which a close contact between the tissues is not effected, the growth of the tumor may still be inhibited at a later period even before the prehatching changes in the allantoic circulation have begun. The inhibition in this case depends upon general changes in the host's mesenchyme called forth by the presence of a growing adult spleen graft on the allantois. The factors of the growth restraint over a tumor grafted in close touch with an adult spleen graft can, therefore, be distinguished as local or direct and general or secondary. Growth restraint is locally brought about by the activity of the groups of grafted splenic mesenchymal cells that come into contact with tumor cells. The general or secondary factors concerned in the inhibition of the growth of the tumor consist in the general changes produced by a spleen graft upon the mesenchyme of the host, of which the allantois is a part. These changes consist in an ubiquitous myeloid metaplasia, as I have described in several papers. In an adjacent double graft the local and general factors of growth restraint are at work together, and it is difficult to distinguish their separate spheres of action. The unfavorable effect of the myeloid metaplasia of the host's mesenchyme on tumor growth can, however, be distinguished from the direct damaging activity of the splenic mesenchyme and studied separately in grafts of spleen and tumor placed at a distance of about 2 to 3 cm. from each other. In this case the much milder inhibition of the tumor growth, though easily noticeable if the myeloid metaplasia is intense, depends exclusively upon the changes in the host's mesenchyme. These secondary factors of inhibition of tumor growth will be analyzed beyond; only the local factors depend-

ing upon the direct activity of the adult spleen are now to be considered.

As soon as contact between the cellular elements of the tumor and adult splenic tissue is established, not only do the tumor cells begin to undergo a profound change, but those of the adult splenic tissue are also affected. In order to appreciate the changes which take place in these tissues in a double adjacent graft, the development and proliferation of a single graft of each of them on the allantois must be borne in mind. As described in the preceding section, one of the most important features of a single tumor graft was an entire absence of reaction between the mesenchyme of the host and the tumor cells. The growth of adult splenic tissue on the allantois has been made the subject of a previous paper<sup>4</sup> and only the more important characters in the process will be mentioned here.

The grafted splenic mash is a mixture of free cells and groups of cells in a syncytial or plasmodial arrangement. The free cells are erythrocytes, wandering cells of various origin, small lymphocytes, scanty granulocytes, and hemoblasts, disconnected endothelial cells and cells of the splenic reticulum. Among them only cells of endothelial and reticular origin proliferate intensely in a splenic graft. These cells display, moreover, an intensive phagocytic and digestive activity against erythrocytes and granular leucocytes. The small lymphocytes leave in great numbers the parts of the graft most remote from the allantois and are found among the mesenchymal cells of the allantois. Conditions here are not very favorable for their further maintenance, and, if not transformed into histiotopic wandering cells, they disintegrate and are ingested by the embryonal mesenchymal cells. The fate of the small foci of the splenic tissue which are not separated into single cells is interesting. Such foci sometimes survive in numbers and become centers of proliferation and further differentiation. The individual cells of the reticulum in such foci hypertrophy, and the tissue, originally in a loose syncytial arrangement, is transformed into true plasmodia with no delimitation of the constituent cells. Many or all of these cells separate as do hemoblasts from blood-islands, and large groups

of free cells arise with the characters of hemoblasts. These cells undergo further differentiation into granuloblasts and granulocytes. Large parts of the adult splenic tissue growing and proliferating in the allantois are transformed into accumulations of granuloblastic tissue. This transformation is greatly favored by a rich ingrowth of vessels and markedly inhibited by the failure of the vessels to develop a sufficiently abundant network.

An adult splenic graft begins to develop in a quite similar manner when grafted on the allantois in close proximity with a tumor graft. Only the granuloblastic transformation of its cellular reticulum is less pronounced in the part of the graft adjacent to the tumor tissue. The cells here retain their fusiform or stellate shape and usually possess numerous slender processes which anastomose with the neighboring cells. A vascular net is regularly found in the peripheral zone of the splenic graft. Numerous round ameboid cells are present in this zone. Hemoblasts and granuloblasts are the most numerous; granular leucocytes are few at the beginning, but more abundant in later stages; small lymphocytes may also be encountered, but they are always scarce—a fact of great interest in connection with the important rôle that has been ascribed to them by Murphy.

The tissue in the extreme outer zone of the splenic graft assumes the form of a loose mesenchyme. This tissue becomes a striking factor not only in limiting the growth of the adjacent tumor graft, but in actively destroying or digesting cell by cell a great part of the tumor tissue. Figure 5 illustrates this process developing in the zone between tumor and spleen graft as soon as the tissues of both grafts come together.

The mesenchyme of the periphery of the adult splenic graft manifests a property entirely lacking in the embryonic mesenchyme of the allantois around a single tumor graft. When single tumor cells grow into this zone they appear morphologically unchanged, but the splenic mesenchymal cells are attracted to them at once. They gather around the tumor cells and accumulate in the recesses between their cytoplasmic processes to form small syncytial groups. These tumor cells still possess

a vigorous metabolism since they continue to proliferate. Tumor cells in mitosis are encircled by mesenchymal cells, even more readily at the beginning, because at this time they lose their cytoplasmic connections with adjacent cells. The first change in a tumor cell around which mesenchymal cells have accumulated is the withdrawal of its processes; it consequently loses its characteristic fusiform or branched shape and becomes spherical. This process has taken place on a large scale in the graft from which figure 5 was drawn. Neither cytoplasm nor nucleus shows at this stage any degenerative change. Numerous cells, however, seem to have become immobile as no cytoplasmic processes appear at their periphery. The splenic mesenchymal cells occur singly or accumulate in small groups around such tumor cells and quickly encircle them, thus separating them from the surrounding tissue. They form an entire capsule around the tumor cells, and include them in small cavities of uninterrupted cellular plasmodium. After being surrounded, the tumor cells undergo a series of physicochemical changes which end only with its complete disappearance. It is gradually transformed by the digestive activity of the surrounding plasmodium into a small mass of non-living protein. The final products of digestion are resorbed and assimilated by the surrounding living cells.

The space within which the tumor cell is found is not a rigid cavity. At the beginning the tumor cell is usually tightly surrounded by the plasmodium; later it lies in a cavity larger than itself, then gradually the cavity becomes smaller, the surrounding cells drawing closer together about the diminishing body of the tumor cell (figs. 7, 9, 11).

The morphological picture of the gradual changes undergone by the tumor cells as seen in the several figures varies greatly. Sometimes the cytoplasm is first attacked, its peripheral zone vacuolized, while a more compact and homogeneous part still remains around the nucleus. In other cases the nuclei seem first to be dissolved, and irregular chromatin particles are found scattered within the cytoplasm. The same parts of the tumor cells do not always disappear first. Sometimes a small chromatic mass, in other cases a clear vacuolized accumula-

tion of metachromatic glassy substance is found as the last vestige of the tumor cell. A quite regular change, and one of the first, is vacuolization of the peripheral zone of the cytoplasm, which loses its normal staining capacity and appears of a purplish and greenish color in Zenker-formol, eosin-azur preparations. A metachromatic purplish staining of the nucleus and of its chromatin is also a frequent occurrence (fig. 15).

When reporting these findings before the New York Pathological Society, I expressed the belief in the destructive power of the adult splenic mesenchyme over the living cell of the Ehrlich sarcoma. Since then further evidence for this has been accumulated. It is not the damaged and weakened tumor cells alone that are surrounded by the splenic mesenchyme, for, as illustrated by figures 5, 9, 12, and 13, cells in all phases of mitosis are encircled in the same way. For some time after being surrounded they seem still capable of maintaining themselves, but they finally succumb within the intraplasmodial cavity.

This destruction of tumor cells by splenic mesenchymal cells checks the further expansion of the tumor graft in the direction of the spleen graft. There is no evidence for a rapid active growth of the splenic mesenchyme within the tumor graft. The reaction described is a necessary sequence of the encounter of two tissues of different specific physicochemical constitution, and although a factor contributing toward the recession of the tumor graft within the allantois of the embryonic organism, it is accidental to the process. The part of the tumor free from contact with the splenic tissue continues to expand for a certain time, and may do so up to the stage at which the prehatching changes occur in the allantois. Its growth may, however, be markedly inhibited at an earlier moment, if the myeloid metaplasia of the allantois called forth by the adult splenic graft is sufficiently pronounced.

The growth contact, and consequently the reaction, between spleen and tumor grafts usually develops at the sixth day, though at times it may be observed on the fourth day, while in other cases it is only slightly indicated even at the eighth day of growth. At this late time the injurious effects of the other

factors, the myeloid metaplasia and the prehatching changes in the allantois, are added. A special series of experiments were required in which the two tissues could be implanted in perfect contact and in which the reaction, if it depended solely on the physicochemical constitution of the two tissues, could develop immediately, before any other factor became active. The account of this series of experiments is given in the following section.

##### 5. GROWTH RESTRAINT OF TUMOR IN A MIXED GRAFT OF TUMOR AND ADULT SPLEEN

It is only the study of mixed grafts of tumor and adult splenic tissue in the allantois which gives full evidence of the phagocytic activity of the adult mesenchyme of the spleen against the tumor cell. The net result of an eight to ten days' growth of mixed grafts in most instances is the full disappearance of tumor tissue, but this fact alone does not permit of any conclusion regarding factors involved in this disappearance. Small lymphocytes, splenic reticular tissue, or even special chemical substances, which may be present in the adult spleen, may equally well be involved in the disappearance of tumor tissue in the double graft. It is necessary to determine whether the tumor foci within the splenic tissue have failed to grow and have become necrotic, or whether their growth has been inhibited and finally stopped by the concomitant growth and differentiation of the splenic tissue, or, finally, whether the tumor tissue has been destroyed by some active process exercised by one of the constituent cellular elements of the spleen. Only a detailed microscopic study of a great number of double grafts, examined at various stages of their development, can furnish definite data concerning these possibilities.

The typical results of one series of mixed graft growths of adult spleen and Ehrlich sarcoma was as follows:

Incubated, 60 eggs; unfertile, 12; grafted, 48; died before the end of the eighth day, 12; no graft developed in 4; 32 grafts were collected, four of which were fixed daily from the second day of growth on.

Grafts of two days' growth: 1. The growth consists of splenic tissue and numerous scattered tumor cells. Some of the tumor cells contain ingested chick erythrocytes and are found occasionally in vacuoles surrounded by mesenchymal cells.

2. Good growth of splenic and tumor tissue. The graft contains two tumor foci surrounded by splenic mesenchyme, partly in loose arrangement, partly in the form of condensed areas. Splenic reticular cells are found in great numbers around the loose tumor cells in the periphery of the tumor focus. Some tumor cells are encircled by patches of small mesenchymal cells; they appear healthy and are either in the resting stage or in mitosis.

3. Intense growth of both tissues. The splenic and the tumor foci are in proliferation. Wherever both tissues come into contact numerous tumor cells are seen to be encircled by mesenchymal cells.

4. The graft as a whole did not take over the greatly thickened serosa. All of the splenic tissue and most of the tumor tissue are necrotic. Groups of tumor cells are found surviving and even proliferating within the necrotic tissue.

Grafts of three days' growth: 1. The graft contains two tumor foci, and a considerable amount of splenic tissue. One tumor focus is growing rapidly and contains innumerable mitoses. It is not completely surrounded by splenic tissue and a zone of marked mesenchymal digestive reaction is present only where both tissues come into contact. Another tumor focus completely surrounded by splenic mesenchyme shows a mesenchymal reaction on its whole periphery. Tumor cells in mitosis are seen encircled by mesenchymal cells.

2. Both tissues in the graft are well intermingled and are in intense proliferation. There are a great number of tumor cells in the resting stage as well as in different stages of mitoses closely surrounded by mesenchymal cells.

3. In this graft the tumor tissue is more abundant than the splenic tissue. Mitoses of tumor cells are very abundant. There is a marked phagocytic reaction wherever both tissues are found in contact.

4. Two foci of growing tumor are found in the graft. Both are surrounded by the splenic mesenchymal syncytium and the process of gradual tumor cell destruction within the mesenchymal capsules is very active.

Grafts of four days' growth: 1. There is a good-sized tumor focus in the midst of intensely growing splenic tissue. The phagocytic reaction is very strong. There are numerous spherical tumor cells in vacuoles, rounded up, with ragged cytoplasm at their periphery. Various phases of degeneration of tumor cells within mesenchymal capsules. Some of the tumor cells, however, are in mitosis, though encircled and in vacuoles; a tumor cell in prophase in a vacuole, and two tumor daughter cells still connected by a cytoplasmic strand encircled by mesenchyme are present. The tumor cells are extensively cut off from the central part of the tumor focus which still has a healthy appearance.



2. The graft contains a large amount of splenic tissue in which a small tumor focus is found surrounded by a large zone of mesenchymal reaction. Thousands of tumor cells are found in vacuoles, greatly altered in the extreme peripheral zone, better preserved toward the central part of the focus. The presence of innumerable vacuoles containing small parts of tumor cells serves as evidence that the tumor growth would have been much more abundant but for the active destruction of tumor cells. In the center of the tumor focus a few healthy tumor cells are present, some of them still in mitotic division.

3. No tumor found. Numerous patches of splenic mesenchymal cells are present.

4. Two tumor foci within a good-sized graft of splenic tissue. One of the tumor foci consists of large isolated tumor cells, practically all of them encapsulated and partly digested. Granular leucocytes are very abundant in this part of the graft. The second of the tumor foci is separated on one side from the splenic tissue by masses of necrotic tissue, and only in this part does the texture of the tumor tissue present a normal appearance.

Grafts of five days' growth: 1. Graft no longer contains tumor tissue. Numerous small accumulations of multicellular plasmodia representing presumably the remains of the condensed mesenchyme after the tumor cells have been digested.

2. The graft is large and contains splenic tissue only.

3. A large graft of splenic tissue contains scanty isolated tumor cells closely surrounded by mesenchymal syncytium, most of them greatly altered.

4. A large graft of splenic tissue, no tumor tissue is present.

Grafts of six days' growth: 1. No tumor tissue is present within a large splenic graft, there are accumulations of plasma cells in the splenic tissue.

2. A large graft of splenic tissue is found in great part necrotic. No tumor tissue is present.

3. No tumor, splenic tissue only, large graft.

Grafts of seven days' growth: 1. Tumor tissue is present in the graft, but only on a part of its circumference is there an evidence of contact with the grafted splenic tissue. Here the digestion of tumor cells has taken place in a most intensive way. In other parts of its circumference the mesenchymal digestive reaction is present, though moderate. The tumor tissue grows actively, while at least at one side it has been destroyed.

2. The graft is large; no tumor tissue is present, splenic tissue is well developed.

3. The graft is large; necrotic in the center. A part of the tissue grafted is found as a necrotic mass above the ectoderm. No tumor tissue is present in the graft. A few tumor cells, abnormal though apparently alive, are found in the necrotic tissue above the ectoderm, thus showing that even under unfavorable conditions the tumor cells may survive seven days while all other cells around them succumb.

4. A remarkably large graft of splenic tissue only is found. In parts of the graft giant-cells and accumulations of condensed cellular reticulum are found, possibly foci of former activity against tumor.

Grafts of eight days: 1. A remarkably large graft of only splenic tissue is present.

2. A very large graft of splenic tissue is found, but no tumor cells.

3. A medium-sized graft of healthy dense splenic tissue is present; there are no tumor cells.

4. A good-sized graft of splenic tissue only is present.

The close analysis of these thirty-two grafts indicates that had all of them been allowed to grow eight days or more, very few would have contained tumor tissue at that period. Only in those mixed grafts, in which the contact between the tumor and splenic tissue had been incompletely effected would tumor still be growing.

The purpose of my further study is an analysis of the factors involved in the disappearance of tumor in grafts in which a complete contact between tumor and adult spleen tissue has been effected at an early period of the graft growth.

The failure to find tumor tissue in a mixed graft of eight days' growth does not depend upon absolute failure of the tumor tissue to grow in such grafts, because at an early stage (two to four days, more particularly three days) actively growing tumor tissue is found. Even in later stages, as for example in a seven day graft, tumor tissue has been found in the graft, but in such cases, even better than in cases of complete disappearance of the tumor, does the effect of the destructive activity of the mesenchyme appear. Only in those parts in which a direct contact of both tissues is effected are the tumor cells, no matter whether in mitosis or in the resting stage, encircled and destroyed. If in contact with the allantoic mesenchyme however, the tumor cells grow unhampered.

The difference between adult splenic mesenchyme and that of the embryonic allantois appears very clearly in these experiments. A positive tropism to the heterogeneous Ehrlich sarcoma cell is shown by the adult splenic mesenchymal cell, and is entirely lacking in the allantoic mesenchymal cell. This affinity of the adult mesenchymal cell, probably of chemical

nature, causes groups of mesenchymal cells to flow together around tumor cells, and separate them from their original connections, encircling them entirely (figs. 5, 7, 9, 11, 12, 13, 14, and 15).

This encircling of the tumor cells is effected gradually and is easily followed microscopically. A rounding up of the tumor cell due to the contraction of its cytoplasm is brought about as soon as mesenchymal splenic cells closely approach it. Tumor cells with long processes at one side may be seen in preparations, while at the other side they are rounded up and in close apposition to a strand of mesenchymal plasmodium. There is, therefore, a marked effect from the approach of the adult splenic mesenchymal cell on the tumor cell, the latter withdrawing its cytoplasmic processes and partly rounding up. At this time it may still be possible for a tumor cell to escape from the zone of the closing mesenchymal ring, since there is yet no indication of a structural change. The steadily and rapidly increasing number of tumor cells entirely encapsulated and cut off from the surrounding tissue, speaks for the inertness of the tumor cell when approached by adult splenic mesenchyme. Of the two, it is the adult splenic mesenchyme which proves to be the more active element, and as a result of this activity the tumor cell is completely immobilized and surrounded by a kind of plasmodial capsule.

Tumor cells only slightly changed or apparently not changed at all, although in vacuoles, are invariably found nearest to the healthy tumor tissue. The fact that the first structural changes which can be detected appear only in tumor cells already encapsulated and that no apparently damaged tumor cells are found outside of the vacuoles within the digestive zone can only be interpreted as evidence for the direct injurious effect of the mesenchymal cytoplasm on a living tumor cell. From the time the tumor cell is situated within the intracytoplasmic vacuole of the mesenchymal plasmodium, its fate is determined. It no longer shows signs of that synthetic metabolism which enables a living cell to maintain itself; it rapidly loses its characteristic cellular structure, gradually disintegrates, and finally disappears entirely.

Even if there existed no information concerning the presence of enzymes in the mesenchymal cells or their derivatives, the study of the gradual disappearance of tumor cells within the mesenchymal capsules would furnish such evidence.

An enzyme working in an acid medium has been demonstrated by Opie in the fowl's spleen. At first glance it would seem difficult to reconcile the results of the present observation with the fact that only an enzyme with proteolytic power in acid medium has been found to exist in the spleen of the fowl; the ground pulp of the organ is approximately neutral ( $\text{pH} = 6.8$ ) when freshly removed from the body. Hedin and Rowland state that the reaction is acid, but their work was done on slaughter-house material, and the methods used were not so delicate as more modern procedures. From the description of the process of tumor destruction it will be seen that actual digestion takes place within closed vacuoles, and that an analogy exists between the activity of the mesenchymal plasmodia against the tumor cells and the well-known digestive power of macrophages known to possess enzymes working in acid medium.

The gradual dissolution of the tumor cells is easily followed within the mesenchymal digestive zone around the actively growing tumor focus. The farther away from the healthy tumor region, the greater are the changes of the tumor cells. In the extreme peripheral zone still recognizable as belonging to the tumor focus in which the tumor tissue is intimately intermingled with the splenic mesenchyme, only very small particles of entirely structureless protein can be discerned. The series of gradual changes from an apparently healthy tumor cell to a small amount of structureless protein can be easily made out in figures 5, 7, 9, 11, 12, 13, 14, and 15.

#### 6. GROWTH RESTRAINT OF TUMOR IN DOUBLE GRAFTS OF TUMOR AND ADULT SPLEEN WIDELY SEPARATED

As shown in the two preceding series of experiments, the tumor cells begin to show regressive changes in their structure only when in direct contact with splenic adult mesenchymal cells. In contrast to the embryonic allantoic mesenchyme, the adult

splenic mesenchymal cell of the fowl manifests a well-pronounced tropism toward the healthy tumor cells of the heterogeneous Ehrlich sarcoma, which enables it to approach the tumor cell or, if the approach be accidental, to keep in touch with it. No immune state is produced by the presence of the adult splenic tissue in the organism of the host, for even that part of the tumor separated from the adult splenic tissue by the width of a few cells only often proliferates as intensely, as if it were grafted alone. The lack of resistance in the embryo is even more apparent in experiments in which the two grafts are placed at a distance from one another of 2 to 3 cm. Under these conditions the tumor graft grows unhampered for about four days, the tumor cells being in contact only with embryonic allantoic mesenchymal cells. Both tissues follow their course of development without any apparent reaction between them.

The graft of splenic tissue, separated from the tumor graft by 2 to 3 cm. grows and develops within the allantois in the manner described in section 4.

The presence of a graft of adult splenic tissue within the allantois soon calls forth extensive changes in all of the embryonic mesenchyme of the host, including the mesenchyme of the allantois itself. These changes as I have described consist in a stimulation of proliferative processes in the mesenchyme. The host's spleen grows to be an organ many times exceeding its normal volume. The delicate strands of connective tissue between the muscle bundles or the nephric tubules, or the testis cords, etc., become transformed into heavy masses of mesenchyme and such derivatives as granuloblasts and granular leucocytes. The allantois itself shows innumerable foci of mesenchyme proliferation. The mesenchymal cells of the vascular adventitia especially proliferate in an intensive way. The proliferating mesenchymal cells are gradually transformed into great masses of ameboid cells—hemoblasts which differentiate into granuloblasts (myelocytes) and granular leucocytes.

At this time the tissue in the tumor graft, when separated from the splenic graft by a distance of several centimeters, acquires an interesting appearance. The embryonic allantoic

mesenchymal cells within the tumor graft and around it proliferate and undergo a myeloid metaplasia just as they do in other localities, but to an even greater extent. At a certain stage the tumor is surrounded and partly infiltrated by a great number of ameboid cells—hemoblasts (fig. 6). These cells were probably described by Murphy as 'round-cell infiltration' and were later mistaken by him for small lymphocytes. The hemoblasts proliferate intensely and numerous mitoses are always present. They also differentiate in great numbers into granuloblasts and finally into the typical granular leucocytes with rod-shaped granulations (fig. 16). As often observed in centers of sudden intensive proliferative and differentiative processes, the typical mode of differentiation shows slight modifications. Leucocytes with spherically shaped granulation are found among the typical leucocytes with rod-shaped granules. With the increasing number of granular cells formed not only locally, but within the whole organism, the infiltration of the tumor tissue by these cells becomes more and more dense. The capillary net is gorged with granular cells. They accumulate in large groups, mechanically forcing the tumor cells far apart (fig. 6).

Though no direct ill effect upon the tumor cells can be observed, their proliferative ability seems to decline, and mitoses are found less frequently in such grafts. It is also of interest that the tumor cells, especially in parts most intensely infiltrated by granular leucocytes, gradually seem to lose the intense basophilic character of their cytoplasm always so evident in centers of rapidly growing healthy tumor tissue. Though directly not affecting the tumor cells, the dense leucocytic infiltration seems to be unfavorable for the growth of the tumor tissue and probably affects to a certain degree the metabolism of individual tumor cell, leaving others, as shown by Stevenson, viable and capable of retransplantation into the mouse. Figures 6 and 16 show parts of tumor tissue at various stages of infiltration by granular cells. Their mother cells have been known as large lymphocytes, but an extensive study of their origin and of their further development has shown them to be in birds and reptiles the common stem cell for all the blood cells (Danchakoff). Maximoff has

found the same to be true for the mammals. Again in figure 6 a number of such stemcells are seen to differentiate into granuloblasts, round acidophilic granules appearing in their cytoplasm. Figure 16 shows to what an extent the granulocytic infiltration can develop. Occasionally tumor cells are found surrounded by heavy capsules of granulocytes. It is rather remarkable that the tumor cells, though seemingly entirely cut off from the nutritive substance by the heavy coats of granular cells, do not easily show regressive changes, as is the case when they are surrounded by mesenchymal cells. This may be explained by the findings of Opie,<sup>16</sup> who proved the leucocytes of the birds to be free from the proteolytic enzymes analogous to that found in the granular leucocytes of mammals. Accordingly, even in the case where leucocytes surround a tumor cell and are retained around it, no marked ill effect on the tumor cell is produced.

No doubt, the intensive granulocytic infiltration does exercise a noticeable restraint on the proliferative ability of the tumor cell in such grafts. It is difficult, however, to decide whether this unfavorable effect is due to a consumption of the available nutritive material by the rapidly proliferating myeloid tissue or, possibly, to certain products of the specific metabolism of the granular cells, which are now added to the milieu in which the tumor cells live.

#### 7. GROWTH OF EHRLICH SARCOMA IN DOUBLE GRAFTS OF TUMOR AND EMBRYONIC SPLEEN

The study of the growth of a single Ehrlich sarcoma graft within the allantoic membrane (section 3) has already shown that tumor cells and those of the allantois at least may live and grow in proximity without any ill effect upon each other. It would not be permissible, however, to assume that all embryonic mesenchyme in contradistinction to the adult splenic mesenchyme has no power to destroy tumor cells. Regional differences may arise in the course of its development and parts of embryonic splenic mesenchyme may acquire properties different from those characteristic of the mesenchyme of other regions of the body. Moreover, the tumor cells in a mixed graft of tumor

and adult spleen might have undergone an injurious effect dependent upon the manipulation of mixing them with another tissue, and, therefore, might in this experiment have become more accessible to the phagocytic action of the splenic mesenchymal cells. A series of experiments seemed necessary in which mixed grafts of tumor and embryonic spleen should be made, in order to obtain direct data concerning the phagocytic and digestive capacity of the embryonic splenic mesenchyme in respect to the growing Ehrlich sarcoma cells.

A preliminary study of single embryonic spleen grafts has shown that its mesenchyme undergoes within the allantois a rapid granuloblastic transformation. While in general similar to the growth processes observed in adult splenic grafts, the changes which take place in the embryonic mesenchyme are more rapid and more extensive. They also differ in certain details. The embryonic tissue of the spleen, comparable to a cavernous tissue, being, in fact, represented practically by the red pulp part of the spleen alone, is always seen in the graft to be supplied by a rich vascular net. The vascular net of the embryonic spleen after grafting rapidly joins with the allantoic vessels, and thus the graft of embryonic splenic tissue ordinarily survives in toto. It appears in sections as strands and islands of mesenchyme traversed and surrounded by large meshes of thin-walled vascular channels. This mesenchyme is seen to proliferate and to undergo a rapid myeloid transformation; granuloblasts and granular leucocytes—products of this metaplasia—find easy access into the lumina of the thin-walled meshes of the vascular net and are at least partly carried away. The granulocytes do not, therefore, accumulate in excessive numbers and do not form large agglomerations as in the adult splenic graft, in which these often undergo a consecutive necrosis. Grafts of embryonic splenic tissue do not in general contain necrotic foci and, what is of interest, do not call forth a myeloid metaplasia in the host's mesenchyme.

In order to study the functional potencies of the embryonic splenic mesenchyme in relation to the phagocytic and digestive capacity, embryonic spleens of the seventh, the fifteenth, and the



twentieth day of incubation were grafted in mixture with Ehrlich sarcoma. The spleen in all these stages is very small, but nevertheless good mixtures were obtained by sacrificing a great number of embryos. The study of such double grafts has shown that they consist of embryonic splenic mesenchyme and tumor tissue intimately intergrown. Strands of mesenchymal cells were seen to traverse the tumor tissue in all directions. Wherever the splenic tissue remained in the graft as larger particles, it underwent a rapid and thorough granuloblastic transformation (fig. 17, lower part). Though in direct apposition with tumor cells, none of the splenic embryonic cells seemed to manifest an activity similar to that displayed by the adult splenic mesenchyme on coming into contact with tumor cells. An infiltration by granular leucocytes in a moderate degree is observed in late stages; it never attains, however, the same degree as in the case of the myeloid metaplasia of the host's mesenchyme, remains always more or less confined to the region in which both tissues come together, and is not followed by any noticeably injurious effect upon the tumor growth. This analysis refers to conjoint growth of tumor and embryonic spleen in all of the stages used, i.e., at the seventh, the fifteenth, and the twentieth day of incubation.

This observation seems to be of particular interest in relation to an inhibiting power on heteroplastic grafting ascribed by Murphy to the small lymphocytes which develop in the spleen in the last stages of incubation. According to Murphy, these small lymphocytes would confer on the whole organism the power of inhibiting heteroplastic grafts in any of its parts. The results of my experiments, in which the embryonic splenic tissue of embryos at the twentieth day of incubation was grafted in close apposition with the tumor, have, however, demonstrated that even the direct contact between the small lymphocytes of the embryonic spleen and the tumor tissue does not exercise inhibiting effects upon the tumor cells.

Other factors than the development of the small lymphocytes must, therefore, determine the striking difference between the results of a tumor-embryonic spleen graft and a tumor-adult spleen graft.

The phagocytic and digestive activity of the adult splenic mesenchyme has been shown to be, in double mixed tumor-adult spleen grafts, the factor of growth restraint or rather of active destruction of two rapidly growing tumors—the Ehrlich sarcoma and tumor 180 (fig. 10). The activity of the mesenchymal cells brings the Ehrlich sarcoma to a rapid disappearance and slows the growth of the 180. The splenic mesenchyme up to the second week after hatching fails to manifest any phagocytic activity against the heteroplastic mammalian tumor cell in mixed tumor spleen grafts. In such grafts the mesenchymal cells do not necessarily approach the tumor cells, neither do they form capsules around them. The results of the two series of experiments are invariably strikingly different: complete disappearance of tumor in the tumor-adult spleen grafts and practically unhampered growth in tumor-embryonic spleen grafts. Since the process of encapsulation and digestion by the adult splenic mesenchyme, in contradistinction to the embryonic tissue, seems to point to an acquisition by the adult splenic mesenchymal cell of a new property lacking in the embryonic splenic mesenchymal cell, further knowledge as to this fact will be gained by an analysis of other means capable of bringing about the same effect.

In these two series of experiments, the same tumor tissue is mixed with splenic tissue in two different developmental stages. As seen from the work of Sherman,<sup>19</sup> the embryo contains at different stages very varying amounts of lysins, opsonins, and complement. The tissue of the adult spleen might, therefore, contain outside its living cells certain substances injurious for the tumor cell, while the embryonic spleen might not contain such substances. Such a possibility cannot be denied without further investigation. If this be the case, the prime factor in the disappearance of the tumor in the double tumor-adult spleen grafts would consist not in a new property of the adult splenic cell, but in some extracellular substances present in the adult, and lacking in the embryo which might produce a change in the heterogeneous mammalian tumor cell and make it more accessible to the phagocytic activities inherent in any mesenchymal cell.

The comparison of the results of these two experiments does not, therefore, necessarily imply a change within the splenic adult mesenchymal cell. The splenic adult mesenchymal cell may remain identical in its functional capacity, as it does in its developmental potencies. The encapsulating and digesting process to which the tumor cell is submitted in a tumor-adult spleen graft would then be determined by a change undergone by the tumor cell, which, though morphologically inappreciable, would nevertheless exert on the otherwise unchanged mesenchymal adult spleen cell a chemotactic effect. In short, the problem reduces itself to the following: Is the encapsulation of the tumor cell by adult splenic mesenchyme due to a definite property of the adult splenic cell acquired by the adult in contradistinction to the embryonic cell, or are the changes of the tumor cells primarily dependent upon the effect of extracellular substances developed in the organism in later stages and present in the adult spleen, but possibly foreign in their origin? If submitted to the influence of such hypothetical substances which might be present in the adult splenic tissue, will the tumor cell then exert upon the embryonic mesenchymal cell a chemotactic action, resulting in the encapsulation of the tumor cell and its subsequent digestion? Another series of experiments was made to clarify this question. Ehrlich sarcoma was mixed with a mash of sterile adult splenic tissue, which had been previously kept frozen, or in a refrigerator for from three to seven days, or in the incubator from one to three days, which, if grafted, would not take. It was expected that if injurious substances were present in the splenic tissue outside the living splenic cells, the tumor cells might be altered in this case in the same way as in the mixed tumor-adult splenic grafts, and that in such a case the embryonic mesenchymal cells of the allantois itself would begin the encapsulation of the tumor cells. Study of such grafts has, however, shown that the tumor grew without showing any injurious effect from the presence of the necrotic adult splenic tissue. The allantoic mesenchymal cells exhibited intensive phagocytic activity against the necrotic particles of the adult splenic tissue, but respected the living Ehrlich sarcoma cells.

No tropism whatever has been observed between the Ehrlich sarcoma cell and the allantoic mesenchymal cell. The tumor cell had not undergone any effect due to the mixing with dead adult splenic tissue, as would have been the case if the changes of the tumor cells in a tumor-adult spleen graft were primarily dependent upon the action of some extracellular substance present in the spleen.

Mesenchymal cells may, however, not be identical everywhere, and may present regional differences in their functional capacities. Splenic mesenchymal cells might be different from allantoic mesenchymal cells, and tumor cells treated by necrotic adult splenic tissue might become accessible for an embryonic splenic cell and not for a mesenchymal cell of the allantois.

Another series of experiments was made in which tumor was grafted in a mixture of necrotic adult splenic tissue and embryonic spleen. It was expected that if the necrotic adult splenic tissue contained extracellular substances which would produce a change in the tumor cell and make it capable of exerting a chemotactic effect upon splenic mesenchyme in general, the embryonic splenic mesenchyme would then exhibit a phagocytic and digestive activity. The study of such grafts, however, failed to reveal any effect of the adult splenic necrotic tissue upon the tumor cells.

In these grafts, as in tumor-necrotic adult splenic grafts, tumor cells themselves occasionally exercise a phagocytic activity against the necrotic splenic adult tissue. It would be of interest to graft tumor with adult splenic tissue, previously thoroughly washed out by saline solution, in order to free it from the presence of any possible injurious extracellular substance and to see whether the phagocytic and digestive activity of the adult splenic mesenchyme against the transplanted tumor cells would still persist.

These two series of experiments clearly show that the adult splenic tissue, frozen or kept at a low temperature from three to seven days or left in the incubator from one to three days, does not contain substances which can change a tumor cell in such a way as to make it exert a chemotactic action on a mesen-

chymal cell either of allantoic or splenic origin. Other experiments have shown that adult splenic tissue submitted for a certain time to a low temperature, but still alive, invariably exhibits a phagocytic and digestive activity against an Ehrlich sarcoma cell. These facts clearly indicate that the prime factor of the encapsulation of the Ehrlich sarcoma cells by a mesenchymal cell must be attributed to the acquisition of a new property by the adult splenic mesenchymal cell. Since the contact with substances outside living cells does not make the tumor cell more accessible for an embryonic allantoic or splenic cell, it must be a change undergone by the adult splenic living cell which makes it capable of encapsulating otherwise uninjured tumor cells.

#### 8. CONCLUSIONS AND DISCUSSION

The above series of experiments and a detailed analysis of their results lead to the conclusion that the adult splenic mesenchyme of the fowl has the power of encircling and encapsulating living Ehrlich sarcoma cells in the allantois of the chick and of submitting such tumor cells in the intracytoplasmic cavities so formed to a process of gradual digestion.

There exists a marked functional difference between the embryonic and the adult mesenchyme of the spleen. The adult mesenchyme of the chick spleen has the power of encircling and digesting not only homologous cells, but also certain heterologous living tumor cells (Ehrlich sarcoma and Crocker Fund sarcoma no. 180), which is lacking in the embryonic splenic mesenchyme; this is not, however, the only distinction between the two different stages of the same tissue. Only a graft of adult splenic tissue on the allantois causes granuloblastic transformation of the host's mesenchyme. The embryonic splenic tissue, even though it rapidly undergoes a granuloblastic transformation, will not call forth any notable change in the host's mesenchyme. This capacity for granuloblastic differentiation is a property common to the splenic mesenchyme of any developmental stage and reveals itself under normal embryonic conditions in only a moderate degree, but can be greatly intensified by

experimental conditions, and can also be awakened anew in the adult splenic mesenchyme by transferring this into the embryonic allantois. The agencies which change the splenic mesenchyme and endow the adult tissue with a capacity of surrounding a living tumor cell and digesting it are not definitely known. Possibly the radical alterations in nutrition which take place after birth are factors in the development of a more vigorous digestive capacity of the adult mesenchyme, but this suggestion at present is no more than a working hypothesis. W. Bullock has found a general change in the organism of the rat which takes place about two weeks after birth, resulting in the development of a resistance against heterogeneous grafting. A few data which have been obtained in regard to the chick allow us to surmise that changes in the mesenchyme of the chick spleen take place at about the same period, for the phagocytic and digestive capacity against heterogeneous cells is not manifested by the grafted splenic mesenchymal cells of animals until two weeks after hatching. It is difficult at present to decide whether the phagocytic and digestive capacity toward heterogeneous living cells is a property which the adult splenic mesenchymal cell develops only within the allantois or whether it may be manifested also in other localities. There exists even less evidence for speculation about the nature of the factors within the allantois which may be especially favorable for the exercise of such an activity. It is not possible at present to determine with any degree of certainty whether the phagocytic digestive property toward a heterogeneous living cell belongs to a mesenchymal cell of the spleen only, or whether it is a general attribute of any adult mesenchymal cell. In mixed grafts of tumor and other organs, the tumor grows unhampered, though a certain amount of adult mesenchyme from the stroma of those organs which are mixed with the tumor tissue is certainly present in these grafts. But if we consider that the stroma cells in organs like the kidney, liver, and muscles are sparse in comparison with the parenchyma of the organ, it becomes obvious that the immediate contact of a tumor cell and mesenchyme will be seldom effected in such grafts, and mesenchymal cells are not in sufficient numbers to encircle and destroy

tumor cells. This capacity may well be a property of the mesenchyme in general, but manifested only by the splenic mesenchyme owing to its greater concentration in the spleen.

The phagocytic and digestive capacity of the adult mesenchyme has long been recognized. Evans' macrophages and Kiyono's histiocytes are isolated mesenchymal or endothelial cells. In many of my own papers illustrations will be found of phagocytic and digestive activity, exercised partly by isolated mesenchymal cells and partly by mesenchyme, the cells of which still retain their syncytial connections.

The mode of ingesting the tumor cells differs to a certain degree from that by which an isolated mesenchymal cell ingests blood corpuscles or any other small fragments of dead material. Adult splenic mesenchymal cells encircle the tumor cell and form a capsule around it, and the tumor cell is finally found situated in an intracytoplasmic vacuole of the plasmodium. The formation of such plasmodia, well known under the name of foreign body giant cells, has long been observed around various kinds of materials. The difference between the phagocytic activity of an isolated mesenchymal cell and that exercised by mesenchymal plasmodia may depend upon the size of the object to be ingested. There must exist, however, a certain positive tropism of the adult splenic mesenchymal cell to the tumor cell, for it has been proved that not every foreign material will exercise such a tactic action upon the mesenchymal and wandering cell. Nor will a definite material like the cells of the Ehrlich sarcoma produce the same tactic effect upon the splenic mesenchyme of different developmental stages.

The results of the present work do not support the recent hypotheses developed by Da Fano, Ribbert, Murphy, and others concerning the immunitive rôle of the small lymphocyte. Murphy states that a bit of adult splenic tissue grafted on the chick allantois together with a tumor, adjacent to it or even at a distance, inhibits the take of the tumor, or, if grafted after the tumor has taken, causes it to recede. He believes the small lymphocyte to be responsible for the 'induced immunity,' though the mechanism by which it effects this immunity remains obscure. The

small lymphocyte, by this hypothesis, is responsible not only for the resistance which is stated to have been obtained against tumor in an embryonic allantois by the introduction of adult spleen, but also for the resistance against heteroplastic grafting which develops naturally in the organism. On the basis of the fact that tumor grafts do not take at the eighteenth to the nineteenth day of incubation, and that a growing tumor recedes at this time, Murphy believed that a general resistance in the chick embryo appears during the last days of embryonic life. He emphasized, however, in his paper the failure to find any noteworthy change in the organism which could account for the appearance of this resistance. He later attributed the resistance to the appearance of small lymphocytes in the spleen of the embryo.

It would seem that this theory concerning the small lymphocyte as bringing about and maintaining an immune state in the organism cannot be sustained. As presented in section three of this paper, the recession of the tumor in the allantois at the eighteenth to nineteenth day of incubation, or its failure to take if grafted at this time, is sufficiently explained by local changes in the membrane itself. At that time these changes affect any tissue, even those from a chick of the same stage as the host itself. It should be remembered also that the spleen, according to Tonkoff, develops at the fourth day of incubation, and that the small lymphocytes, according to Danchakoff, begin their differentiation in this organ at about the fifteenth day of incubation. Small lymphocytes, however, appear in the blood current from about the twelfth day of incubation because of their development in the thymus. No directly injurious effect upon the tumor cells, however, by any element of the spleen of animals younger than one to two weeks has ever been observed. All this tends to show that the development of the general resistance of the chick against heterogeneous grafting cannot be demonstrated at a period before birth, that in no case is the appearance of the small lymphocyte in the organism responsible for the development of such a resistance, and that up to two weeks after incubation none of the cellular elements of the embryonic spleen is capable of either inhibiting tumor growth or injuring tumor cells.



The fact that W. Bullock found no resistance against heterogeneous tumor in the rat until two weeks after birth is very significant, for it is at about the same time that the splenic chick mesenchyme develops its characteristic phagocytic and digestive power over the mammalian Ehrlich sarcoma cell. Whether this coincidence should be regarded as accidental or as a consequence of common factors only further research can show. Bullock found that a graft of adult splenic tissue in a new-born rat at a distance from the tumor graft did not result in an induced immunity in the young animal. His results, therefore, though relating to mammals, do not support the lymphocyte hypothesis.

Murphy further states that he has regularly obtained inhibition of a tumor implant if a bit of adult spleen were grafted together with the tumor. "Round-cell infiltration" was observed by him around the necrotic tumor tissue, the "round-cells" being later described as small lymphocytes. Stevenson repeated these experiments, using the same tumor, and regularly obtained a conjoint growth of tumor and spleen. As seen in section four of this paper the tumors employed grew fairly well in double grafts, grafted adjacently or at a distance. Stevenson proved the presence of living tumor cells within grafts by re-grafting them back into mice, and he also described an infiltration of the tumor by granular leucocytes. This infiltration is only one of the expressions of a general myeloid metaplasia which develops in the mesenchyme of the host after adult splenic grafts. These changes in the host depending upon a graft of adult spleen on its allantois seem not to have been observed by Murphy.

The failure in Murphy's experiment of the tumor to take if grafted adjacent to the splenic tissue or even at a distance is difficult to understand, unless he used a sarcoma in its regressive phase, when a dense infiltration with granulocytes might have produced a sufficiently injurious effect upon it to check the growth.

There is also little to say in regard to the analogy which has been drawn between the resistance observed in the adult animal

and that supposed to have been developed in the embryo when it was supplied with a small bit of adult lymphoid tissue. "The lack of resistance seen during the early days of the incubation period is replaced by a degree of resistance comparable with that observed in the adult animal if the embryo is supplied with a small bit of adult lymphoid tissue" (Jour. Exper. Med., 1918, vol. 28, no. 1). But it is obvious that no analogy can be drawn between an adult organism with its well-developed resistance against heterogeneous grafting and an embryo into which a small bit of lymphoid tissue has been introduced, for such an embryo does not manifest resistance. The destructive power of the adult splenic cell toward the Ehrlich sarcoma cell is the attribute of an adult mesenchymal cell, just as general resistance is an attribute of the adult organism.

The digestive capacity of the adult splenic mesenchyme toward the Ehrlich sarcoma cell, though interesting in itself, cannot necessarily be connected with the natural resistance of the host against heteroplastic grafting. It has not yet been observed under ordinary conditions in animals naturally immune or artificially immunized, the reported evidences of phagocytosis being confined entirely to dead tumor cells. The fact, however, that this digestive capacity is called forth in the mesenchyme some time after birth and at the same time that the general resistance is developed may ultimately prove to be of great importance in a further analysis of the factors determining the changes in the embryonic splenic mesenchyme and conferring on it a digestive capacity.

Though not necessarily having a causal relation to the natural or artificially developed resistance of the organism, the mesenchymal digestive activity might be at least partly responsible for it. As a tissue, the mesenchyme is the least differentiated and the collagenous fibers which it develops are not accumulated within the cytoplasm, but are cast off outside. The cytoplasm of an adult mesenchymal cell undergoes little change and may never lose any of the fundamental properties of the cell, by which I mean a further differentiative ability, proliferation, and digestion of particulate matter as do many of the other cells of

the organism. Its proliferative power, which is revealed under favorable conditions, is well established, also its intracellular digestive capacity. Though this digestive property of the mesenchyme has been recognized as widely distributed in the animal kingdom, it has not yet been made an object of special inquiry in relation to the equally widely distributed resistance against heteroplastic grafting. Researches along these lines promise to reveal new facts which may bring a better understanding of the special case of the digestive activity exercised by the adult splenic mesenchyme of the fowl on the cells of two mouse tumors, the Ehrlich sarcoma and the Crocker Fund sarcoma no. 180, a process which at present stands as a unique phenomenon.

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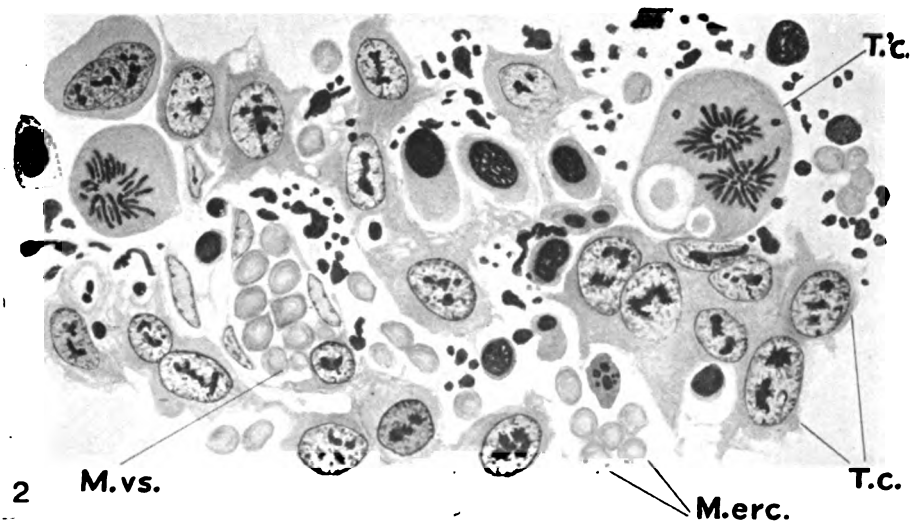
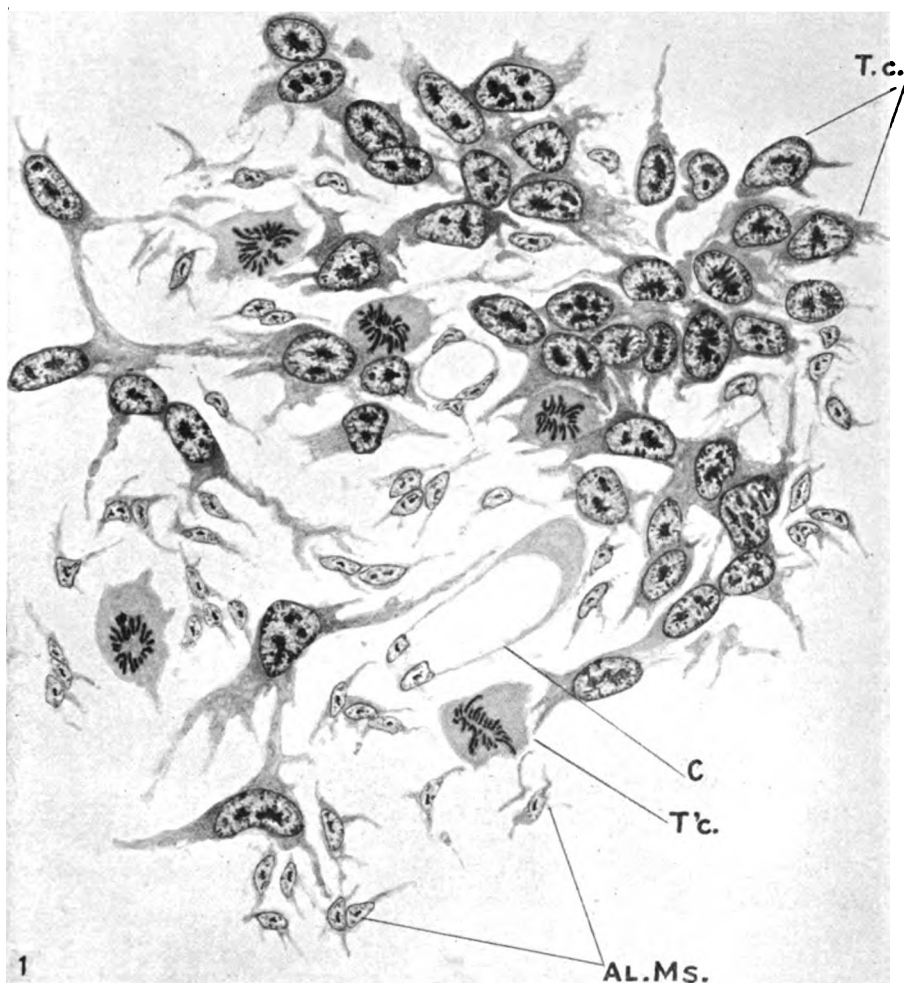
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## PLATE 1

### EXPLANATION OF FIGURES

1 Section through a part of Ehrlich sarcoma graft grown four days on the chick allantois. Tumor cells loosely arranged at the periphery of the graft and proliferating intensely. The small embryonic allantoic mesenchymal cells and vessels grow between the groups of tumor cells *Al. Ms.*, allantoic mesenchyme; *c*, capillary; *Tc*, tumor cells.

2 A section through an Ehrlich sarcoma graft which remained separated by the serosa from the allantoic mesenchyme, forty-eight hours after grafting. Most of the tumor cells, *Tc*, show a normal structure and two are seen in mitosis, one of them has ingested two mouse erythrocytes; *M. erc.*, mouse erythrocytes still well preserved. The endothelium of the mouse vessel *M. vs.* is in regression.

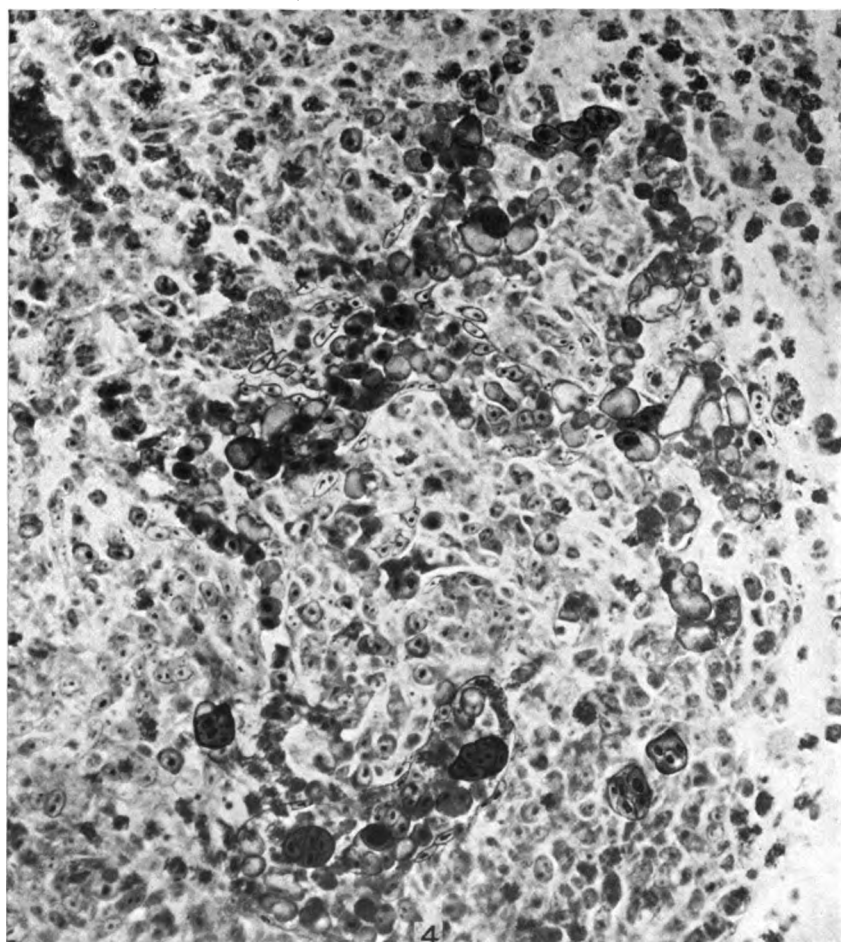
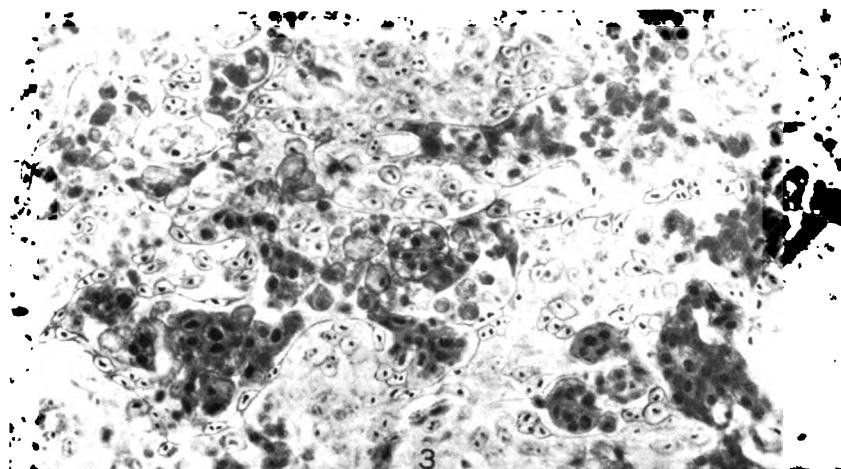


## PLATE 2

### EXPLANATION OF FIGURES

3 Capillary network in an adult spleen graft on the fifteenth day of host incubation and on the eighth day of the graft growth. The blood within the capillaries is stagnant and undergoes regressive changes. Macrophages are present and show numerous ingested erythrocytes.

4 Capillary network in an adult spleen graft on the eighteenth day of host incubation and on the eleventh day of graft growth. The blood within the capillaries shows further regressive changes. Numerous erythrocytes flow together and are seen in the form of huge structureless blocks, the nuclei disappearing and the cytoplasm gradually losing its hemoglobinic reaction. Macrophages are seen very active in ingesting the disintegrated erythrocytes. Retouched photographs,  $\times 400$ .

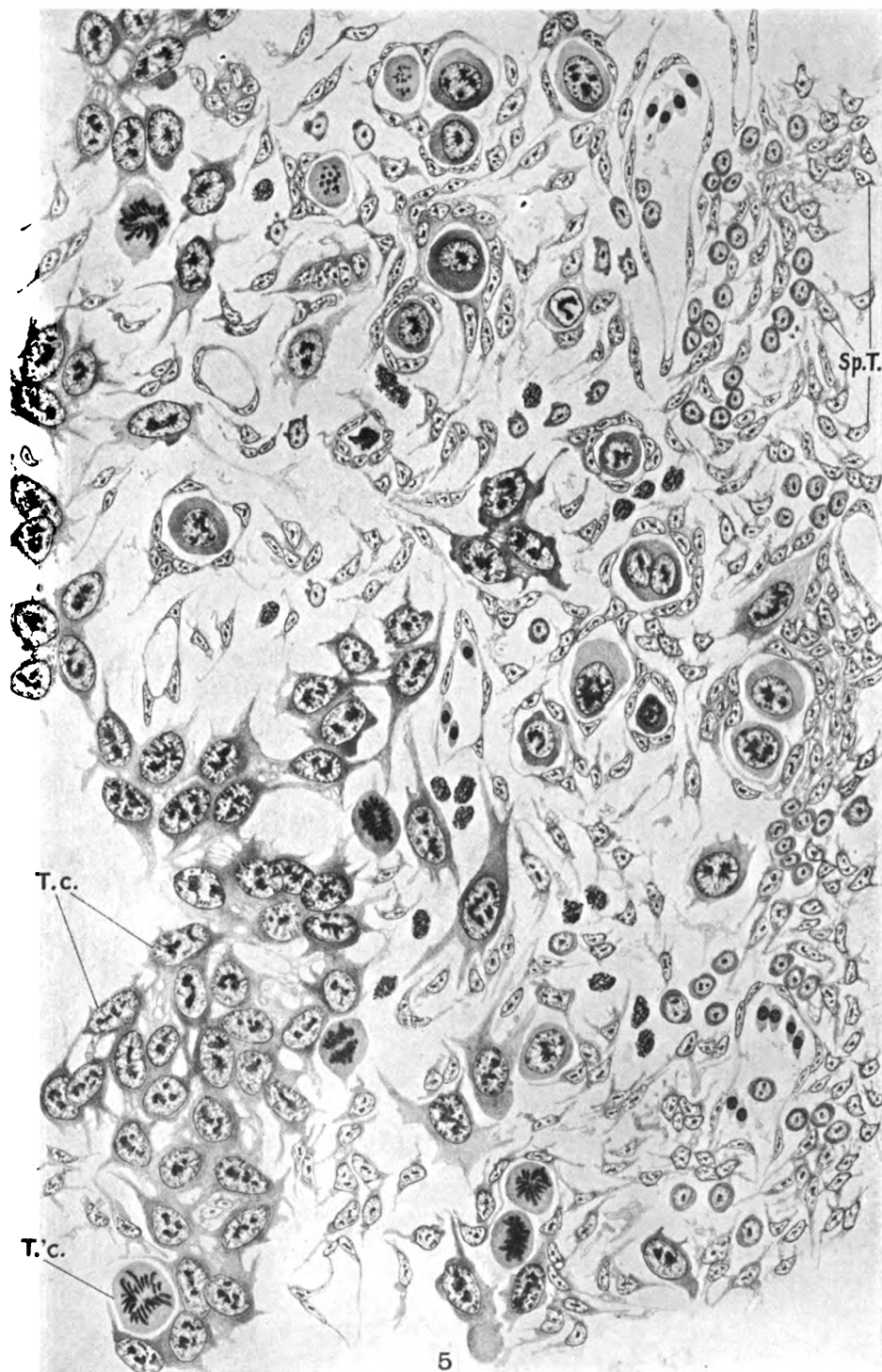




### PLATE 3

#### EXPLANATION OF FIGURE

5 Section through a part of adjacent tumor (Ehrlich sarcoma)—adult spleen grafts after six days of conjoint growth. The left side of the figure is occupied by healthy proliferating tumor tissue. The right side of the figure is occupied by the splenic tissue. In the region where the two tissues are in contact numerous tumor cells are seen surrounded by mesenchymal cells which form a capsule around them. The tumor cells within the vacuoles appear almost spherical, some of them showing regressive changes. *Sp. T.*, adult splenic tissue; *T.c.*, tumor cells.

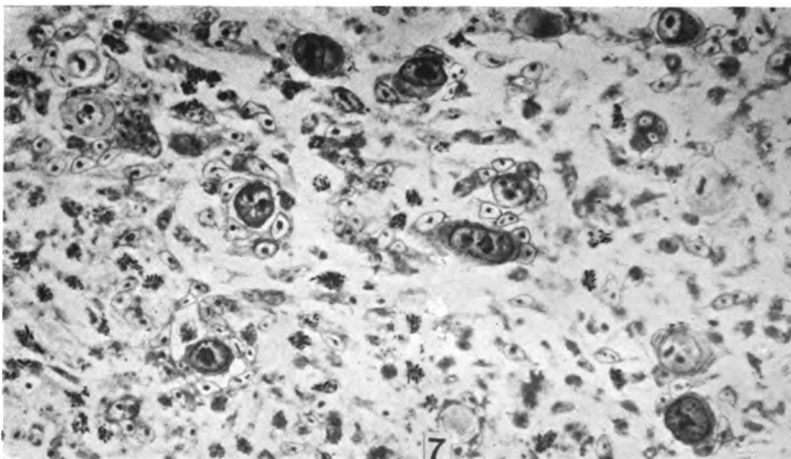
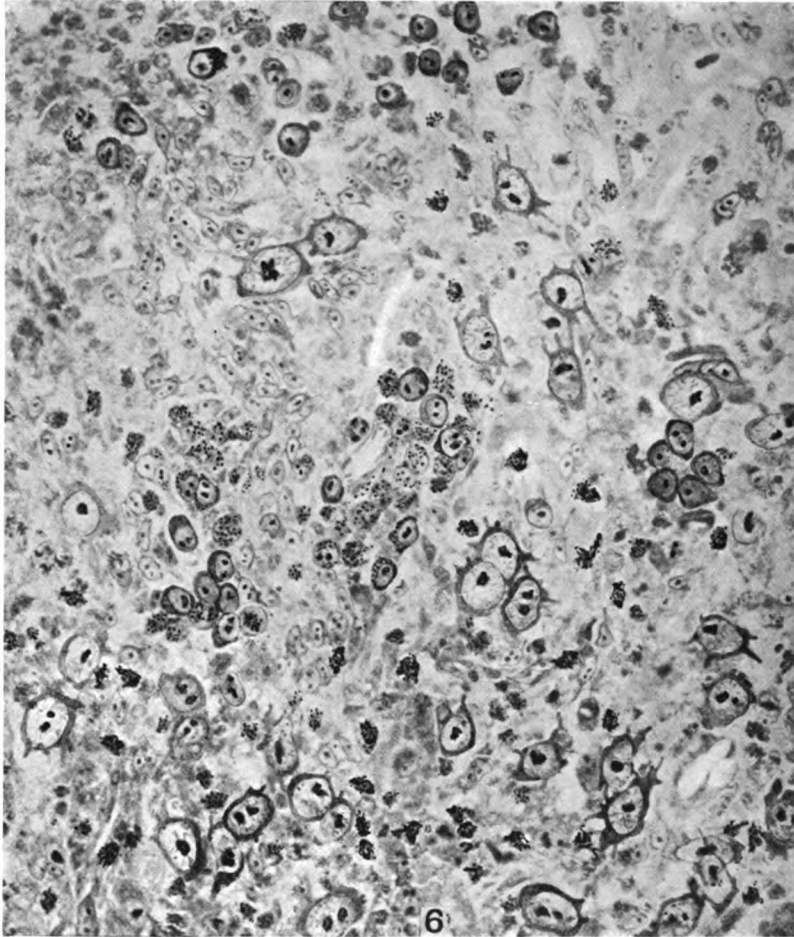


## PLATE 4

### EXPLANATION OF FIGURES

6 Section through adjacent tumor (Ehrlich sarcoma)—adult spleen graft after seven days of conjoint growth. The figure represents the part of the graft not in immediate contact with the adult spleen tissue. A granuloblastic metaplasia in the entire mesenchyme of the host took place as a result of the splenic graft. The tumor cells are seen separated by heavy strands of mesenchyme and by a large number of 'round cells,' which in this particular case are hemoblasts, granuloblasts, and granulocytes.

7 Section through a mixed tumor (Ehrlich sarcoma)—adult spleen graft after four days of conjoint growth. Only single tumor cells are found, all of them surrounded by mesenchymal capsules, many of them showing well-pronounced regressive changes. Retouched photographs.  $\times 400$ .

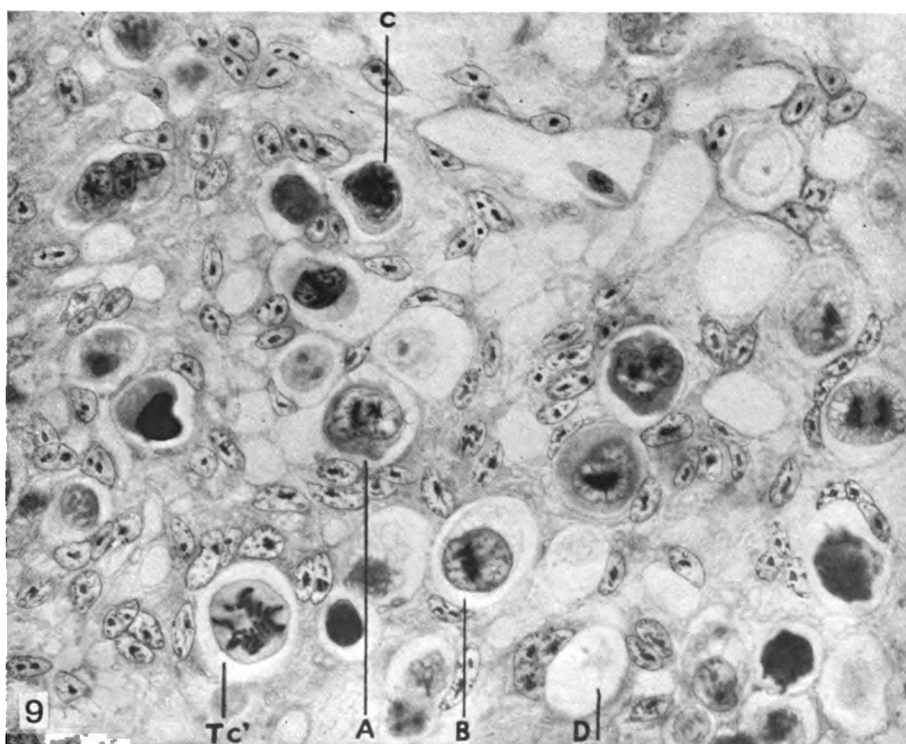
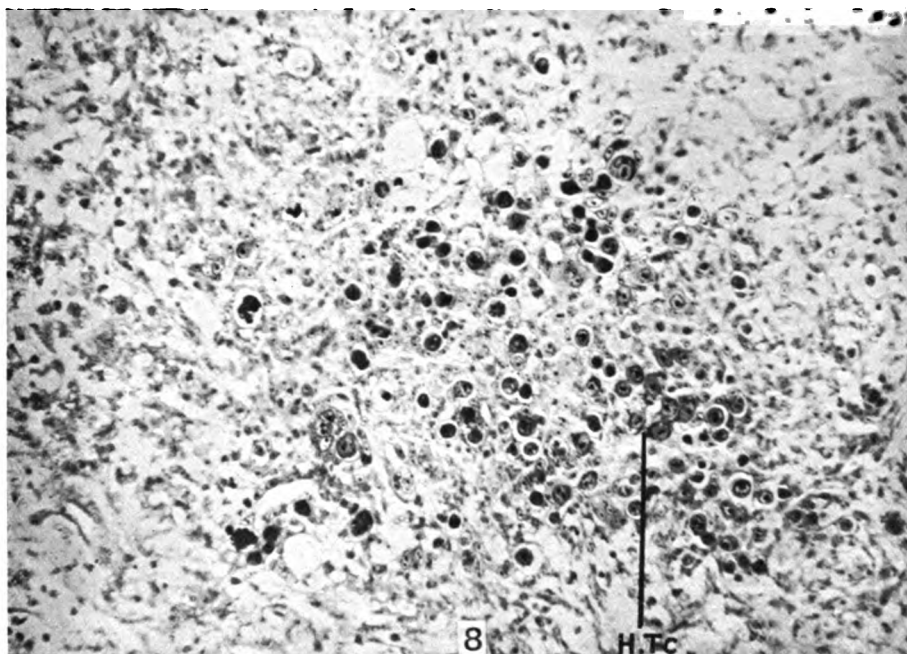


## PLATE 5

### EXPLANATION OF FIGURES

8 Section through a mixed tumor (Ehrlich sarcoma)—adult spleen graft after four days of conjoint growth. Only a very small tumor focus is found. Practically all the tumor cells, except for a few of them (*H. Tc.*), are in vacuoles, surrounded by mesenchymal capsules.

9 Large magnification, 1000 diams. of a small area of figure 8. *Tc'*, tumor cell in mitoses. *A, B, C, D*, show different stages of gradual digestion of tumor cells.

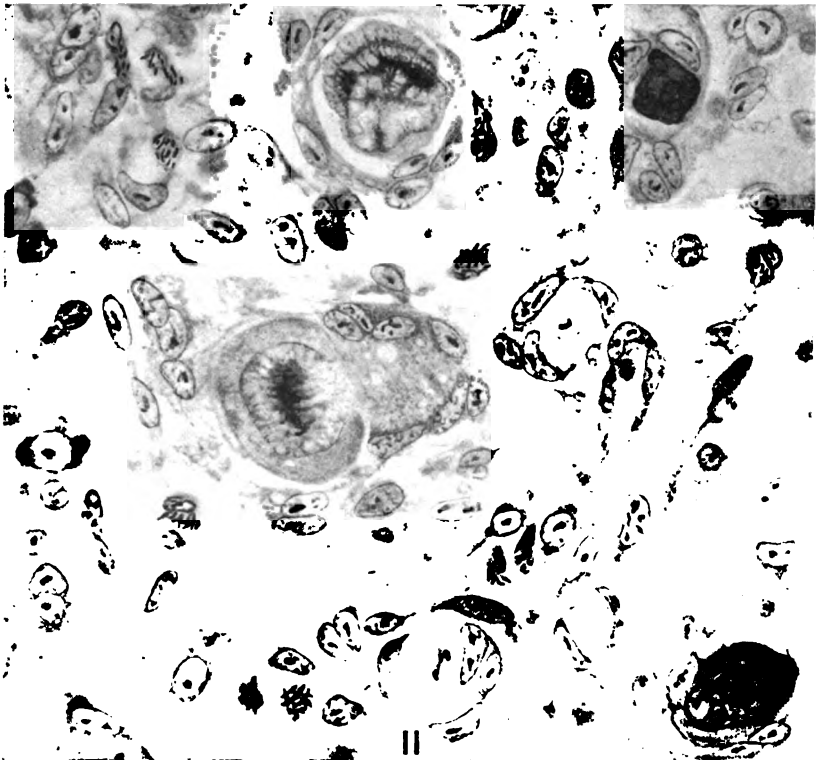
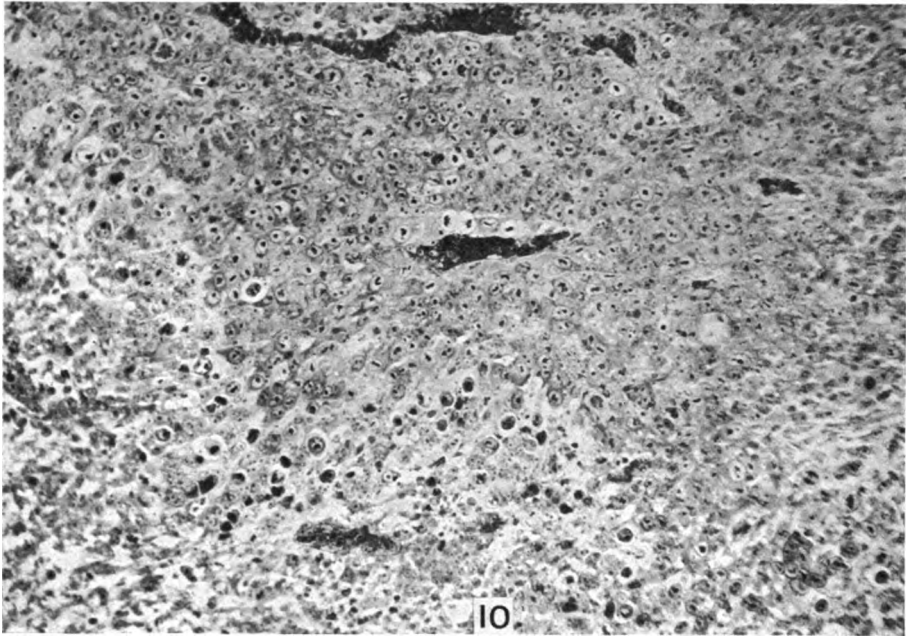


## PLATE 6

### EXPLANATION OF FIGURES

10 Section through a tumor (180)—adult spleen graft after five days of conjoint growth. The tumor tissue intensely proliferating, but around it a zone is found in which the mesenchymal cells are surrounding the tumor cells forming cellular capsules and digesting them in the so-formed vacuoles.

11 Large magnification,  $\times 1000$ , of a small area of figure 10. The tumor cells in the vacuoles are seen in different stages of digestion.





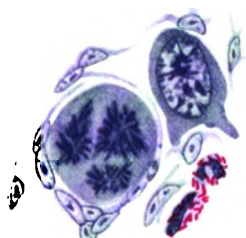
## PLATE 7

### EXPLANATION OF FIGURES

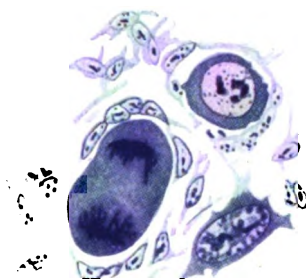
12 and 13 Tumor cells in mitoses surrounded by mesenchymal capsules in a mixed Ehrlich sarcoma—adult spleen graft.

14 Tumor (Ehrlich sarcoma) cells surrounded by mesenchymal capsules, some of them still showing normal structure except for their spherical shape.

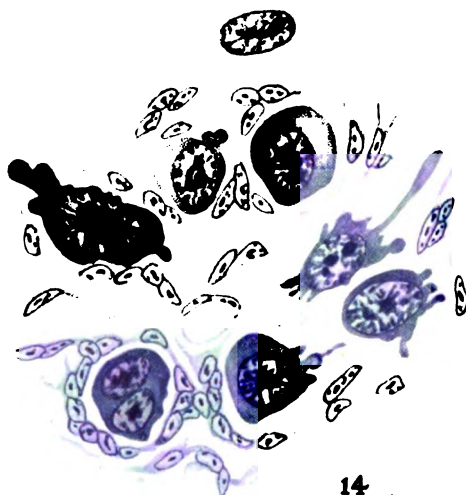
15 Gradual changes of the tumor (Ehrlich sarcoma) cells within the mesenchymal capsules, after four days of growth in a mixed tumor adult spleen graft.



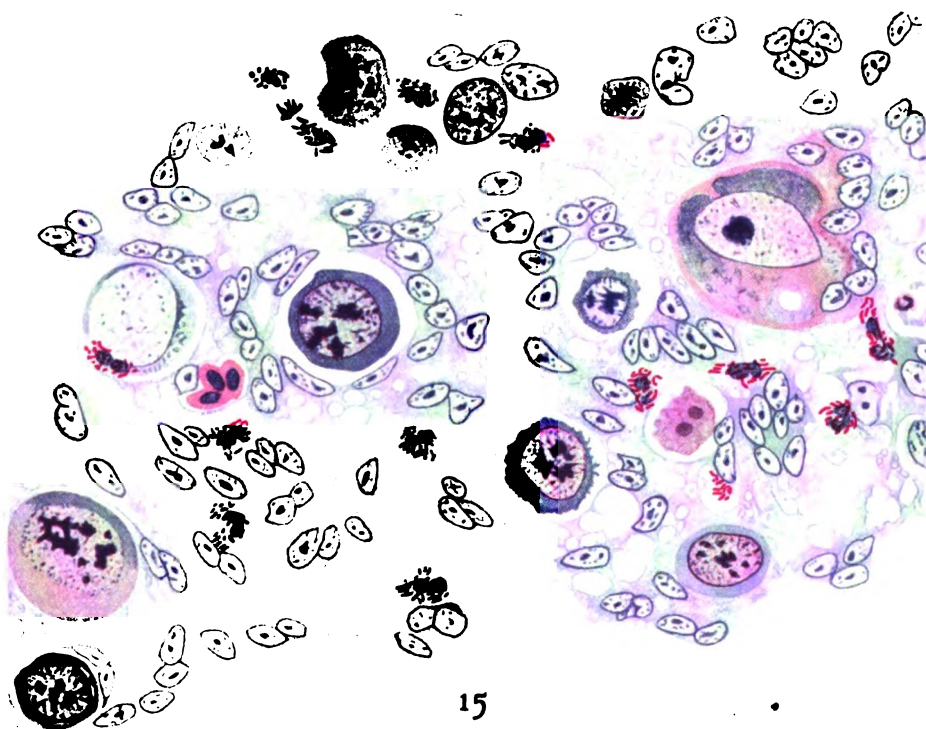
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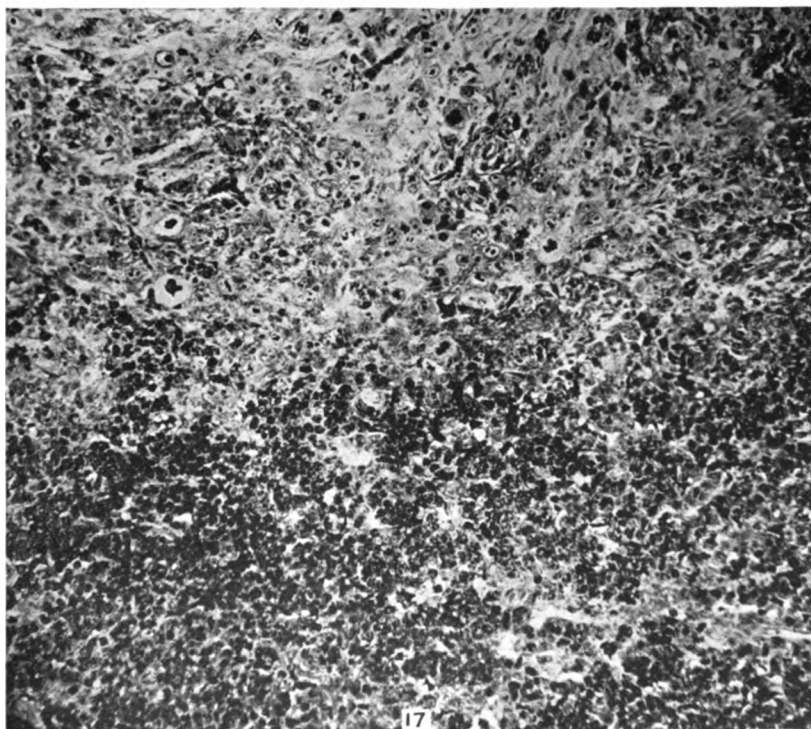
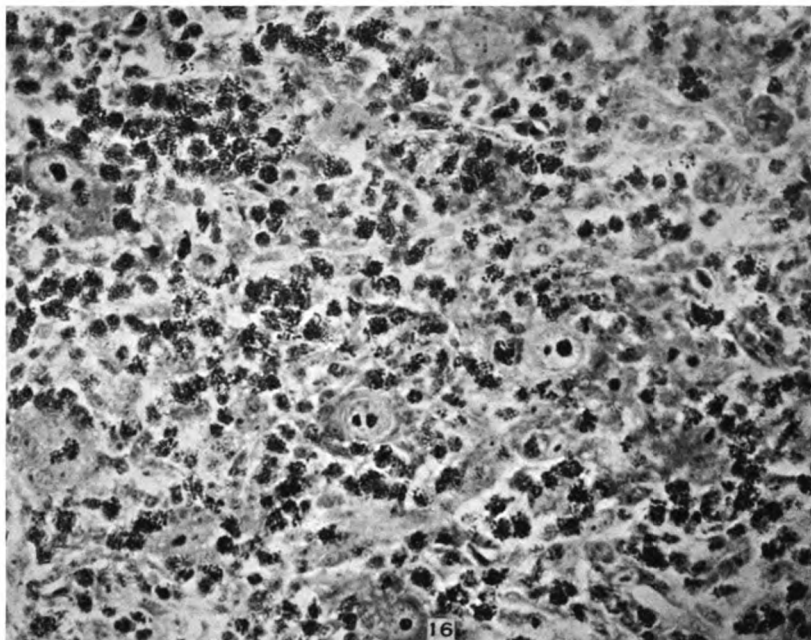
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## PLATE 8

### EXPLANATION OF FIGURES

16 Section through a tumor graft, grown eight days on the allantois conjoint with an adult splenic graft at a wide distance. The tumor tissue is seen intensely infiltrated by granular leucocytes, the tumor cells widely separated, but still occasionally found in mitosis.

17 Section through a tumor (Ehrlich sarcoma)—embryonic spleen graft six days after conjoint growth. The embryonic splenic tissue has undergone an extensive granuloblastic transformation, the tumor tissue is moderately infiltrated by granular leucocytes. No digestion zone develops between the two tissues.





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